LIPID PEROXIDATION AS AN ALTERNATIVE PATHWAY FOR BILIRUBIN METABOLISM

Thesis submitted to the University of London for the degree of Doctor of Philosophy.

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ABSTRACT

A standard hepatic microsomal model for the peroxidation of membrane phospholipids was found to degrade bilirubin to colourless diazo-negative products. Lipid peroxidation (LPO) and bilirubin degradation (BRD) activities were strongly correlated and both showed identical requirements for cofactors and oxygen, regardless of whether peroxidation was initiated enzymatically or non-enzymatically. Bilirubin did not appear to have an antioxidant effect on LPO in the system tested. Superoxide anions, hydroxyl radicals and hydrogen peroxide were not found to have a significant role in microsomal BRD activity and are probably not the oxidants involved in the enzymatically driven, NADPH-dependent LPO studied. Lipid radicals arising during LPO are most likely the oxidising species involved in BRD.

In order to examine the possible role of LPO in maintaining bilirubin homeostasis in the bilirubin UDP-glucuronyl transferase deficient Gunn rat, plasma bilirubin was modulated by altering the diet. A lipid free diet caused a significant rise in plasma bilirubin (BR), together with marked decreases in microsomal LPO and BRD activities, compared to a high lipid diet which resulted in a decrease in hyperbilirubinaemia and increased LPO and BRD activities. Neither of these diets influenced the microsomal ethoxyresorufin deethylase activity, suggesting that cytochrome P_{448} was not involved in the dietary effect. However, the high lipid diet resulted in a higher proportion of unsaturation in the fatty acid chains of the microsomal phpspholipid compared with normal or lipid free diets, which could account for the increased LPO.

In vivo injection studies with two antioxidants were performed. Vitamin E, abolished LPO and BRD but did not alter plasma bilirubin concentration over 3 days. Vitamin C, had no effect on LPO and BRD and a variable effect on plasma bilirubin concentration.

Both *in vitro* and dietary studies suggest that the oxidation of bilirubin by LPO might provide an alternative pathway for bilirubin disposal in Gunn rats.

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ABBREVIATIONS

BR - bilirubin IX_α

$BR^{\cdot}$ - bilirubin radical

NADPH - ß-nicotinamide adenosine dinucleotide phosphate (reduced form)

BSP - bromosulphthalein

ICG - indocyanine green

UDP-GT - uridine diphosphate glucuronyl transferase

UDPGA - uridine diphosphate glucuronic acid

LPO - lipid peroxidation

ATP - adenosine 5'-triphosphate

ADP - adenosine 5'-diphosphate

AMP - adenosine 5'-monophosphate

$HO_2^{\cdot}$ - hydroperoxy radical

PUFA - polyunsaturated fatty acid

LOOH - lipid hydroperoxide

$L^{\cdot}$ - fatty acid alkyl radical

$LOO^{\cdot}$ - conjugated peroxyl radical

$LO^{\cdot}$ - lipid alkoxyl radical

LH - polyunsaturated fatty acid

$O_2^{\cdot -}$ - superoxide anion

SOD - superoxide dismutase

EDTA - ethylene diamine tetracetic acid

GSH - reduced glutathione

LDL - low density lipoprotein

DMSO - dimethyl sulphoxide

Cyt. P_{450} - cytochrome P_{450}

AMVN - 2,2'-azobis (2,4-dimethylvaleronitrile)

AAPH - 2,2'-azobis (2-amidinopropane) hydrochloride

MOPS - 3-[N-Morpholino] propane sulphonic acid

TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin

DBI- double bond index

PLFA - phospholipid fatty acid

TG - triglyceride

FFA - free fatty acid

PL - phospholipid

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CHAPTER 1

INTRODUCTION TO BILIRUBIN METABOLISM

1.1 BILIRUBIN METABOLISM

1.1.1 ORIGIN

Bilirubin IX_α (BR) is the major catabolite in the degradation of haemoglobin in man and other mammals. Approximately 70% of the BR produced is derived from haemoglobin of senescent erythrocytes that have been removed from the circulation by the reticuloendothelial system mainly in the spleen but also in the bone marrow. The red cell is engulfed and lysis occurs inside the phagocytic cells which then degrades the liberated haemoglobin. The red cell in man has an average life span of 120 days and in the rat of 60 days. In subcutaneous bruises, tissue macrophages degrade the haemoglobin to bile pigments, which accounts for the colour changes from dark purple (haem) to blue-green (biliverdin) and eventually to yellow (bilirubin).

In haemolytic disease, the sinusoidal cells of the spleen and liver (Kupffer cells) play a major role in the sequestration and degradation of immunologically or chemically damaged erythrocytes. In intravascular haemolysis the hepatic parenchymal cells and renal tubules may become important sites for the conversion of circulating haemoglobin-haem to bile pigments, which is not bound to haptoglobin.

In addition to red cell haemoglobin, 20 % of the daily bilirubin production of

approximately 300 mg in man comes from the rapid turnover of hepatic and extra hepatic haem containing enzymes (eg. cytochrome P_{450}, cytochrome b_5, catalase and tryptophane pyrrolase) and about 10 % from ineffective erythropoeisis in the bone marrow.

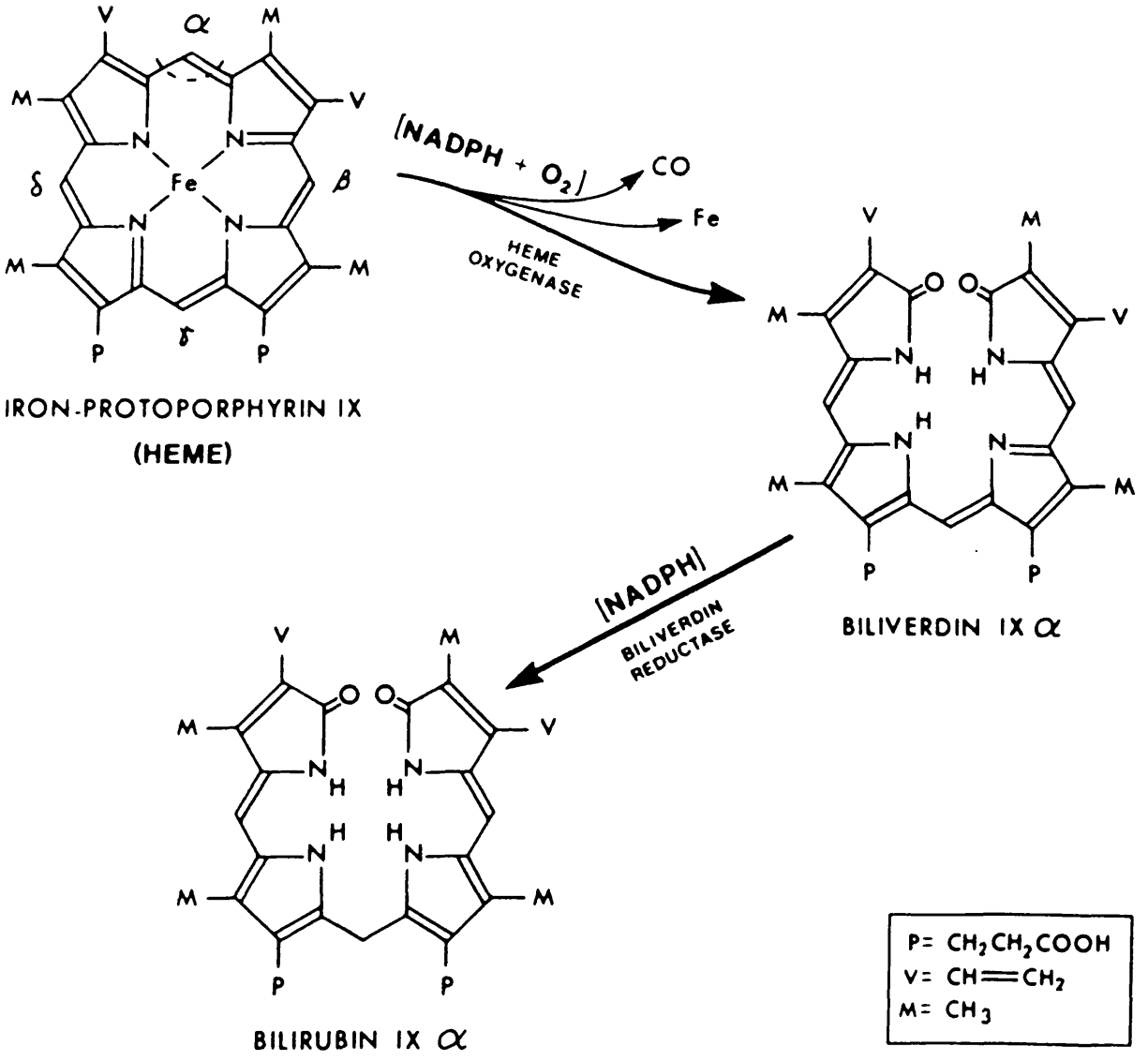
1.1.2 FORMATION

It has long been debated as to whether haem catabolism *in vivo* is a non-enzymatic process or an enzymatic process. Although the two mechanisms are probably not mutually exclusive (Schmid & McDonagh,1975), current thinking favours the microsomal haem oxygenase system which consists of reduced nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome P_{450} reductase and haem oxygenase (Fig 1.1) (Crawford et al, 1988). Since haem proteins are not good substrates for the enzyme *in vitro*, it seems likely that *in vivo* the protein moiety is first degraded and the haem binds to the haem oxygenase in the membrane of the endoplasmic reticulum. The enzyme is substrate inducible. In the presence of NADPH and molecular oxygen the iron protoporphyrin is converted to mesohydroxy haem and is then oxidatively cleaved at the α-meso-bridge carbon atom so that carbon monoxide is released; the iron is transferred to iron binding proteins and is reutilised, and the green pigment biliverdin IX_α is formed. In mammals biliverdin IX_α is reduced at its central methene bridge to bilirubin IX_α by the cytosolic NADPH dependent enzyme biliverdin reductase. Bilirubin is released into the blood, where it is bound to plasma albumin.

1.1.3 STRUCTURE OF BILIRUBIN

The naturally occuring bilirubin isomer is not a linear tetrapyrrole as is conventionally illustrated but rather an involuted structure that is maintained by six intramolecular hydrogen bonds between the carboxyl groups of the propionic acid side chains and the amino and lactam groups of the opposite halves of the molecule (Bonnett et al, 1976, Fig 1.2). In this conformation, the hydrophilic groups are shielded and bilirubin is insoluble in water. Esterification with a sugar moiety at one or both propionic acid side chains decreases intramolecular hydrogen bond formation and hence greatly enhances water solubility.

FIG. 1.1 FORMATION OF BILIRUBIN



1.1.4 TRANSPORT IN BLOOD

Bilirubin is transported in the blood from its site of production to the liver, where it is converted to a water soluble derivative (conjugated bilirubin). The solubility of unconjugated BR at a physiological pH is verv low and therefore its transport to the liver involves binding of a small quantity to red blood cells while the majority of the bilirubin is bound to albumin (Jacobsen,1969). The albumin molecule has two classes of binding sites for bilirubin. Under physiological conditions, bilirubin in plasma is almost completely bound to albumin at the high affinity binding site and only a very small fraction is present as unbound bilirubin. It is assumed that the unbound bilirubin is the fraction which enters the tissues and when in excess may produce toxic effects, particularly in the newborn brain (Brodersen,1980).

Conjugated bilirubin is also transported in blood bound to albumin, although its affinity for albumin is less than that for unconjugated bilirubin so that it can be excreted in bile and urine.

1.1.5 HEPATOCELLULAR TRANSPORT

Uptake

Unconjugated bilirubin is rapidly removed from the circulation by the liver. The mechanism of transfer of bilirubin from albumin to hepatocytes is not well understood. The existence of a specific albumin receptor has still to be demonstrated. Specific plasma membrane binding proteins for bilirubin have, however, been isolated and there is good experimental data to support the concept that bilirubin, bromosulpthalein (BSP) and indocyanine green (ICG) are transported by a specific carrier system, which is different from that involved in hepatic transport of bile acids and fatty acids (Sorrentino & Berk, 1988). Hepatic uptake of bilirubin has been shown to be a carrier-mediated, saturable process that obeys Michaelis-Menten kinetics and is shared by several other organic anions such as BSP and ICG, which exhibit competitive inhibition (Scharschmidt et al, 1975).

The results of studies with radiolabelled bilirubin in jaundiced Gunn rats and normal rats indicate that the rate of hepatic uptake of bilirubin is greater than that of the excretion of conjugated bilirubin in normal bile and is therefore independent of this process (Scharschmidt et al, 1975).

Conjugated bilirubin is taken up by the liver at a much faster rate than unconjugated bilirubin; the mechanism of uptake seems to be very similar since it shows competitive inhibition with unconjugated bilirubin and other organic anions (Shupek et al, 1978).

Intra cellular transport

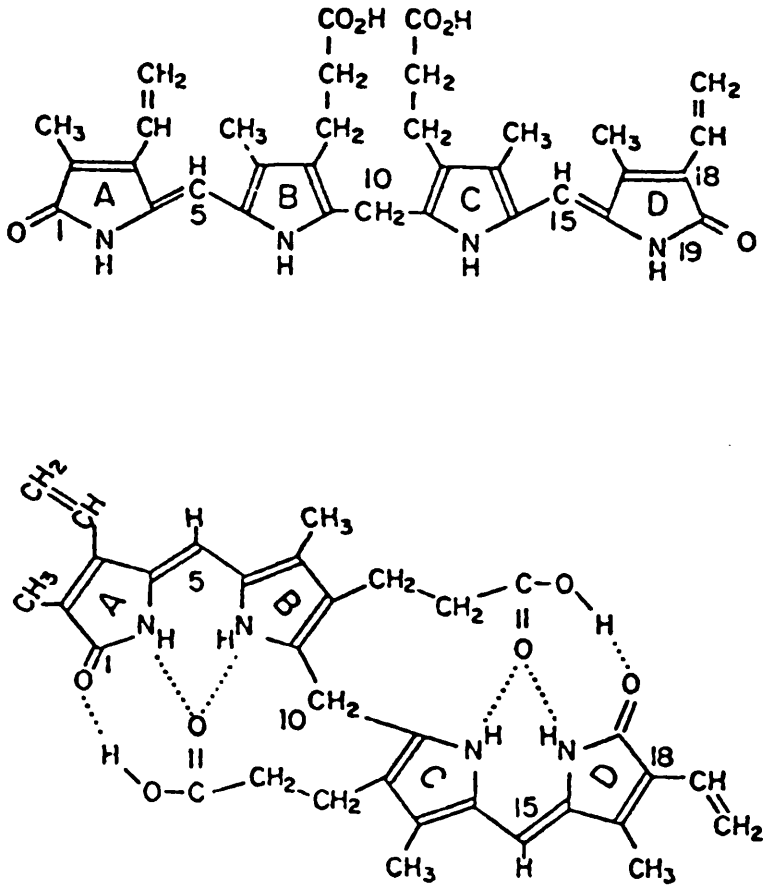
Once bilirubin has entered the hepatocyte it is bound in the cytosol to ligandin (Y protein) and to a lesser extent to Z protein. Ligandin also binds a wide range of other substrates such as haem, carcinogens and hormones (Bhargava & Arias, 1981). The hepatic content of ligandin has, however, been found not to affect the influx of the pigment but it does reduce the efflux from the liver and Wolkoff et al (1979) have postulated that this is the main role of ligandin in hepatic transport. Bilirubin transport within the cell is highly compartmentalized since bilirubin produced from hepatic haemoproteins appears to follow hepatic channels which differ from those carrying exogenous pigment from plasma to bile (Krishenbaum et al 1976).

1.1.6 CONJUGATION

Bilirubin requires to be converted to a polar derivative before it can be excreted in bile or urine. This involves esterification of one, or both, of the propionic side chains attached to C8 and C12 with a glycosidic moiety so that hydrogen bonding of the bilirubin molecule is reduced (Fig 1.3). Glucuronic acid is the major conjugating sugar in normal mammalian bile pigments. The diglucuronide constitutes 70-80% of the total bile pigments in human bile and the C8 and C12 monoglucuronide isomers are present in approximately equal amounts.

The formation of the monoglucuronide is catalyzed by the membrane-bound

FIG.1.2 STRUCTURE OF BILIRUBIN



Bilirubin IX α is internally hydrogen bonded between the carboxyl groups of the propionic acid side chains and the amino and lactam groups of the opposite halves of the molecule, indicated by dotted lines. (Bonnett et al 1976).

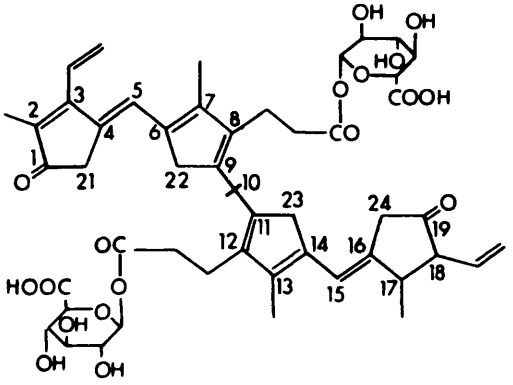
enzyme hepatic bilirubin UDP-glucuronyltransferase (UDP-GT) which is primarily located in the rough and smooth endoplasmic reticulum with smaller amounts in Golgi membranes and the nuclear envelope (Hauser et al, 1984). The enzyme transfers the glucuronyl group to bilirubin from its co-substrate, uridine diphosphate glucuronic acid (UDPGA). Conversion of the monoglucuronide isomers (C8 and C12) to the diglucuronide is also mediated by UDP-glucuronyltransferase, although this activity of the enzyme *in vitro* appears to be considerably lower than that for monoglucuronide synthesis.

It has been postulated that bilirubin UDP-glucuronyl transferase consists of four subunits (Peters et al,1984). A single subunit can catalyse the glucuronidation of bilirubin to bilirubin monoglucuronide whereas the complete tetrameric enzyme is required for the conjugation of bilirubin to the diglucuronide.

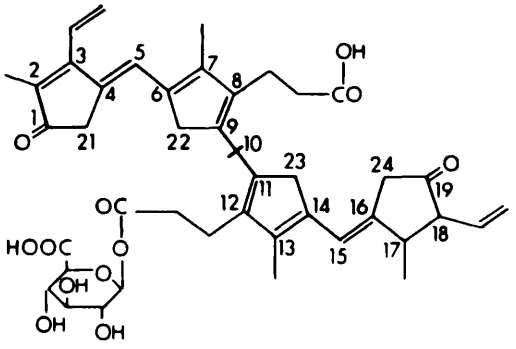
Bilirubin UDP-GT is a member of a family of UDP-glucuronyltransferases which catalyse the glucuronidation of many compounds including drugs such as morphine, lorazepam and chloramphenicol, potentially toxic products such as cannabinoids, benzopyrene, phenols and endogenous compounds such as thyroxine, sex steroids and certain bile acids. The liver contains numerous UDP-glucuronyltransferases with overlapping specificities for these various substrates. A specific bilirubin-conjugating form of the enzyme has been isolated, which catalyses not only glucuronidation, but also glucosidation and xylosidation of bilirubin (Chowdhury et al, 1986). These findings are consistant with the observation that in patients with the Crigler-Najjar syndrome type I and in the Gunn rat animal model, bilirubin glucuronidation is not detectable *in vivo* or *in vitro* whereas glucuronidation of other substrates is unimpaired or only slightly reduced. In the Gunn rat, bilirubin UDP-GT mRNA is absent (Burchell et al, 1987).

The various glucuronyltransferases develop at different rates. Bilirubin UDP-glucuronyltransferase belongs to the 'neonatal' group of transferases, which do not develop until after birth and then gradually increase towards adult levels (Wishart, 1978). It is induced by cytochrome P_{450} related drugs such as phenobarbitone rather than cytochrome P_{448} related drugs such as 3-methyl-cholanthrene.

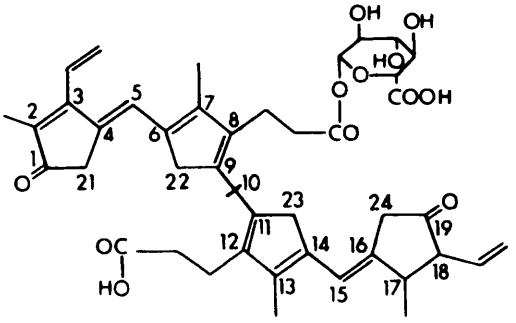
FIG. 1.3 BILIRUBIN GLUCURONIDES



BILIRUBIN DIGLUCURONIDE



BILIRUBIN MONOGLUCURONIDE (C-12)



BILIRUBIN MONOGLUCURONIDE (C-8)

Both propionic acid side chains are glucuronidated in bilirubin diglucuronide. Bilirubin monoglucuronide can exist as two molecular species, depending on whether the C12 or C8 propionic acid is conjugated.

The activity of glucuronyltransferases appear to be profoundly influenced by the lipids of the microsomal membrane in an unknown way. This activity *in vitro* can be enhanced by detergents such as digitonin or Triton X 100, sonication or treatment with phospholipases (Zakim & Vessey, 1975; Berry et al 1978). Purification of the enzyme may produce a preparation that lacks lipids and this leads to greatly diminished activity, which can be restored by the addition of phospholipids (Burchell 1980). The type of phospholipid and the length and degree of unsaturation of the fatty acids appear to be important in the reconstitution.

It has been demonstrated *in vitro* that a small fraction of the bilirubin glucuronides formed in the liver undergoes hepatic deconjugation (Cuypers et al, 1984). To what extent this mechanism is of physiological importance remains to be established.

1.1.7 BILIARY EXCRETION

The mechanism by which bilirubin glucuronides are transferred from the endoplasmic reticulum to the canalicular membrane of the hepatocyte has not been defined and may possibly involve an intracellular vesicular pathway. Similarly, little is known about the transport of bilirubin glucuronides across the canaclicular membrane. It has however, been established that the transport of bilirubin from plasma into bile proceeds against a concentration gradient and is carrier mediated since the biliary concentration of bile-pigments exceeds that in plasma up to 40 fold. It is probably energy dependent and operates at a lower capacity than either uptake or conjugation (Forker, 1977). The biliary excretion of bilirubin glucuronides is believed to be the rate limiting step in the overall transport of bilirubin from blood to bile.

The canalicular bilirubin carrier system appears to be shared by a variety of other organic anions such as bromosulphthalein and rose bengal which compete with conjugated bilirubin for biliary excretion, although more than one membrane carrier may be involved. Bile acids such as taurocholate form micelles and increase the excretion of bilirubin and other organic anions (Forker, 1977). It has been postulated that within the canalicular lumen the micelles of bile acids, phospholipid

and cholesterol sequester conjugated bilirubin so that the concentration of 'free' conjugated bilirubin is reduced; this favours the secretion of bilirubin (Scharschmidt & Schmid, 1978). Bile contains only small amounts of unconjugated bilirubin (< 1 %), even when there is a defect in conjugation as in Gilbert's or Crigler-Najjar syndromes (Fevery et al, 1977); under these conditions the total excretion of bile pigments in bile is significantly reduced and the proportion as monoconjugates increased.

1.1.8 METABOLISM IN THE INTESTINAL TRACT

Conjugated bilirubin reaches the large intestine unchanged and then deconjugation occurs and the bilirubin moiety is reduced by bacteria to a group of bile pigments called urobilinogens. The structure of the urobilinogens formed depends on the flora of the gastrointestinal tract (Watson, 1959). The majority of the urobilinogen/urobilin pigments formed are excreted in the faeces although some reabsorption occurs. There is also an enterohepatic circulation for unconjugated bilirubin. Its physiological significance in the adult is uncertain, although it may be important in neonatal hyperbilirubinaemia (Brodersen & Herman, 1963) and in patients on antibiotic therapy.

1.1.9 RENAL EXCRETION

In the normal subject and patients with unconjugated hyperbilirubinaemia bile pigments are not present in the urine. However, in patients with cholestasis, urinary excretion is the main route for the disposal of conjugated bilirubin (Fevery et al 1967).

1.2 HYPERBILIRUBINAEMIA

In normal plasma, the total bilirubin concentration ranges between 5 and 17 μM, and varies directly with bilirubin turnover and inversely with hepatic bilirubin clearance (Bloomer 1971).The term hyperbilirubinaemia can be used to indicate

an abnormally high plasma concentration of either conjugated or unconjugated bilirubin or both. In normal subjects, virtually all the plasma bilirubin is in the unconjugated form. In contrast, circulating bilirubin in patients with hepatocellular or biliary tract disease consists predominantly of mono- and di conjugates.

Unconjugated hyperbilirubinaemia may be caused by (1) haemolysis or ineffective erythropoiesis both of which accelerate pigment formation, or by (2) abnormal hepatic function which can involve impaired hepatic blood flow, defective hepatic uptake or impaired conjugation of the pigment.

Conjugated hyperbilirubinaemia predominates in diffuse hepatocellular injury (eg. hepatitis or cirrhosis) where there is the likelihood of defects both in hepatic transport and in biliary secretion. Impaired bile secretion at either the canalicular or bile duct level results in cholestasis and subsequent conjugated hyperbilirubinaemia, due to regurgitation of the pigment into the blood. The unbound conjugated bilirubin is excreted in urine.

The following discussion will consider only those disorders in which the unconjugated hyperbilirubinaemia is attributable to a congenital defect.

1.2.1 NON-HAEMOLYTIC UNCONJUGATED HYPERBILIRUBINAEMIA

1.2.1.1 Crigler-Najjar syndrome, type I (Crigler & Najjar, 1952)

This is an extremely rare form of severe unconjugated hyperbilirubinaemia. It is associated with neurological dysfunction, and appears between the first and third days after birth and persists throughout life. The syndrome is generally fatal during the first months of life, although on rare occassions patients have survived beyond puberty (Blumenschein et al 1968). It is due to a genetic defect of bilirubin glucuronyltransferase, which seems to be inherited as an autosomal recessive trait (Arias et al 1969). Plasma bilirubin concentrations usually exceed 340 μM, all the pigment being in the form of unconjugated bilirubin. Unconjugated bilirubin, which normally constitutes less than 1 % of bile bilirubin, accounts for more than 30 % of the total bile pigment, which is markedly decreased (Fevery et al 1977). Most

bilirubin produced by such patients is excreted in bile or urine in the form of polar, colourless diazo-negative derivatives (Schmid & Hammaker,1963). Phenobarbitone and other microsomal inducing agents fail to stimulate bilirubin glucuronide formation or to reduce plasma bilirubin concentrations (Arias et al,1969; Berk et al, 1975).

Experimental approaches to treatment of hyperbilirubinaemia

A variety of experimental approaches to lowering bilirubin concentration, and thus preventing bilirubin encephalopathy, have been explored. Continuous **extracorporeal perfusion of blood through an affinity chromatography column** containing an albumin-conjugated agarose gel has proved successful in reducing the hyperbilirubinaemia of Gunn rats; however, major problems relating to the biocompatibility of the system need to be overcome before this approach can be applied to humans. **Plasmaphoresis** has proved efficient in removing substantial amounts of bilirubin, but, since the fall in plasma bilirubin is transient, it is hardly a practical treatment on a long-term basis. It has however produced dramatic clinical results in some patients with rapidly deteriorating neurological signs. **Oral administration of binding agents** such as agar or cholestyramine which has proved of some value in neonatal jaundice (by inhibiting reabsorption of the small amount of unconjugated bilirubin in the intestine) has not been consistently beneficial in Crigler-Najjar patients. **Phototherapy** has been successful in reducing plasma bilirubin levels. Maintainance of an adequate response, however, requires about 12 hours exposure each day (McDonagh & Lightner, 1985). Also, occasional cases are resistant to phototherapy and treatment becomes ineffective after several years of intense light exposure. **Enzyme replacement** is another possibility, which needs to be further explored. Since the normal liver contains bilirubin UDP-glucuronyltransferase, transplantation of this organ should produce a dramatic fall in plasma bilirubin concentration and has been performed in a few cases (Kaufman et al, 1986).

A procedure that may have important applications in the clinical treatment of neonatal jaundice has been described and involves the use of a highly specific enzyme to remove bilirubin from the bloodstream. The **fungal enzyme**, isolated

from Myrothecium verrucaria, converts bilirubin to biliverdin and other products less toxic than bilirubin.

The enzyme has been covalently bound to an agarose column and when the blood of Gunn rats was passed through the serum bilirubin concentration decreased by 50 % in 30 min (Lavin et al, 1985).

1.2.1.2 Animal model - Gunn rat

A similar disorder to the Crigler-Najjar Type I syndrome, is present in a jaundiced mutant strain of Wistar rat, first reported by Gunn (1938). The phenotypic inheritance pattern is consistant with that of a single recessive gene. Gunn rats have a complete absence of bilirubin glucuronyltransferase activity and fail to respond to phenobarbitone treatment (Schmid,et al 1958, Cohen et al, 1985). Their livers are histologically normal and excretion of other organic anions is only slightly reduced or normal. The hepatic uptake and intracellular binding of unconjugated bilirubin is normal (Bernstein et al, 1966). The presence of kernicterus, similar to that found in man, has been reported in some rats (Johnson, et al 1959).

1.2.1.3 Crigler-Najjar Syndrome, type II.

Patients with the Crigler-Najjar syndrome, type II exhibit an intermediate degree of hyperbilirubinaemia due to a partial defect in bilirubin conjugation. Liver function tests and histology are normal and these patients have no signs of haemolysis. Jaundice is the only abnormal physical sign; intellectual or extrapyramidal impairment rarely develops even when the plasma unconjugated bilirubin levels exceed 340 μM for many decades (Gollan et al, 1975). Bilirubin encephalopathy is much less frequent than in the type I syndrome. The total biliary pigments in these patients are significantly lower than normal and there is a predominance of bilirubin monoglucuronide (Fevery et al, 1977).

Phenobarbitone and other microsomal enzyme-inducing agents produce a dramatic fall in plasma bilirubin concentration in contrast to patients with Crigler-Najjar type I syndrome (Arias et al, 1969, Black et al, 1974). It is generally thought

that these drugs act by inducing bilirubin glucuronidation.

However, increased hepatic bilirubin UDP-glucuronyltransferase activity has not been demonstrated, possibly due to lack of sensitivity of the assay.

1.2.1.4 Gilbert's Syndrome (Gilbert & Lereboullet, 1901)

This is a relatively common, benign syndrome and is characterized by mild fluctuating unconjugated hyperbilirubinaemia while other liver function tests such as serum transaminases, alkaline phosphatase, albumin and bile acids are normal. Gilbert's syndrome occurs in up to 7 % of the population (Berk et al, 1975) and is thought not to be associated with overt haemolysis or any histologic or biochemical evidence of liver injury. This condition predominates in males (Bailey et al 1977). Plasma bilirubin concentrations usually range from 20 to 90 μM. These levels are increased two to three fold by fasting, reduction in caloric intake, intravenous glucose or an intravenous injection of nicotinic acid which appears to increase erythrocyte fragility and thus bilirubin formation.

Gilbert's syndrome is usually diagnosed in the late teens or early twenties, often following a blood test taken for some other purpose since clinical icterus is not always apparent. The traditional approach to the diagnosis of Gilbert's syndrome requires the exclusion of increased bilirubin production by haemolysis. It is, however, now known that reduced red cell survival and Gilbert's syndrome may coexsist so that up to 60 % of patients have a mild state of haemolysis or dyserythropoiesis. Haemoglobin is usually normal and reticulocytes are not raised.

The pathogenesis of Gilbert's syndrome is a matter of controversy, mainly because no single defect readily explains all the observed abnormalities. It is now well established that hepatic bilirubin clearance is reduced to about 30 % of normal and that UDP glucuronyltransferase activity is decreased. These findings considered together with the observations that microsomal enzyme inducers decrease plasma bilirubin concentration and normalize the abnormal biliary pigment pattern, are all consistant with the hypothesis that reduced glucuronidation is the main factor responsible for the decreased hepatic bilirubin

clearance and resultant hyperbilirubinaemia.

However, analysis of plasma disappearance curves after radioactive bilirubin administration has suggested that the majority of patients with Gilbert's syndrome also have a moderately reduced rate of hepatic bilirubin uptake (Black et al, 1974). Also, a significant reduction in the plasma disappearance rate of BSP or ICG, neither of which is conjugated with glucuronic acid, has also been observed in some patient sub groups. These latter findings cannot be explained by a decrease in bilirubin glucuronyltransferase activity and suggest a generalised defect in the hepatic uptake of organic anions.

1.3 ALTERNATIVE PATHWAYS FOR BILIRUBIN METABOLISM IN THE CRIGLER - NAJJAR SYNDROME AND GUNN RATS.

Both patients with Crigler-Najjar syndrome type I (Schmid & Hammaker, 1963; Arias et al, 1969) and Gunn rats (Schmid et al, 1958) have a complete deficiency of microsomal bilirubin UDP-glucuronyltransferase activity which is inherited as a homozygous recessive trait. This prevents them from excreting bilirubin as water soluble conjugates and as a result they both have a severe unconjugated hyperbilirubinaemia. Despite this defect in bilirubin metabolism, the raised bilirubin concentration in the blood remains relatively stable although haem continues to break down at a normal rate. This indicates that equilibrium has been attained between production and elimination of bilirubin.

Schmid and Hammaker (1963) used C14 labelled bilirubin to examine this problem and showed that, in Gunn rats and a Crigler-Najjar patient, disappearance of the radioisotope from the plasma was significantly slower than normal and that bilirubin was converted to colourless, polar diazo negative compounds which were excreted mainly in the bile (and faeces), with small amounts of label in the urine. Their findings indicated that catabolism of bilirubin had occured in the liver and gastrointestinal tract.

Schmid and Hammaker (1963) also detected considerable radioactivity in the bile and faeces of rats with biliary fistula, probably as the result of the transport of unconjugated bilirubin across the intestinal wall, prior to degradation by bacterial flora. Recent experiments by Kotal and Fevery (1990) have demonstrated that urobilinogen, which is formed from bilirubin by this route and is then reabsorbed and excreted in bile, constitutes one of the major bilirubin derivatives of freshly analysed Gunn rat bile; its formation cannot however account for all of the bilirubin eliminated.

Small amounts of hydroxylated bilirubins have been detected in Gunn rat bile. This suggested the involvement of microsomal cytochrome P_{450}-dependent monoxygenase(s) in the alternative pathway of bilirubin metabolism (Berry et al, 1972). Support for this hypothesis came from the experiments of Kapitulnik and Ostrow, (1977) who treated jaundiced Gunn rats with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent inducer of cytochrome P_{448}. After 4 to 6 days, the plasma bilirubin concentration decreased by 61 % due to a seven-fold increase in fractional bilirubin turnover and was associated with an increased excretion of polar bilirubin metabolites. Phenobarbitone, which is an inducer of a different cytochrome P_{450} isomer did not influence the plasma bilirubin level unless the Gunn rats were also subjected to phototherapy (Cohen et al, 1985). It is of interest that the microsomes of both newborn and adult jaundiced Gunn rats have a higher content of cytochrome P_{448} compared to the non-jaundiced heterozygotes (Kapitulnik et al, 1987).

Various attempts have been made to define an *in vitro* enzymatic system capable of oxidising bilirubin. Brodersen & Bartels (1969), following the observation that bilirubin rapidly disappeared from the brain of a child with kernicterus, reported the presence of an insoluble bilirubin oxidase in the mitochondria of guinea pig and human brain, which required oxygen for activity. The products of the reaction were not characterized but included biliverdin and a diazo negative polar pigment.

Further support for the concept that a mitochondrial oxidase might be involved in the catabolism of bilirubin came from the studies of Yokosuka and Billing (1980), who demonstrated significant enzymatic activity in homogenates of the kidney and

intestine of the Sprague-Dawley rats. Cardenas-Vasquez et al (1986) then demonstrated the presence of the enzyme in hepatic mitochondria following subcellular fractionation. In view of the induction studies with TCDD (Kapitulnik & Ostrow, 1977), it was to be expected that the microsomal fraction of the liver would play an important role in the degradation of bilirubin. Indeed, since this work was begun, studies by De Matteis et al (1989) have demonstrated such a system which requires NADPH and oxygen and is associated with the cytochrome P_{448}/NADPH - dependent reductase system.

Finally, Kaul et al (1980), injected rats intraperitoneally with bilirubin and then added xanthine in order to stimulate generation of superoxide anions by xanthine oxidase. They showed that treated rats had a shorter half life of plasma bilirubin than rats that did not receive xanthine and suggested that superoxide radicals might act as mediators of bilirubin degradation *in vivo*.

The most salient feature of the investigations of the alternative pathway for bilirubin metabolism in the Gunn rat model of congenital unconjugated hyperbilirubinaemia, is that an oxidative mechanism is implicated. Metabolites have been identified as hydroxylated and polar compounds and the involvement of a specific enzyme of the microsomal monoxygenase system has been suggested.

Peroxidation of microsomal membrane lipids could be a possible mechanism for the alternative pathway for bilirubin metabolism since it is an oxidative process which gives rise to reactive radical intermediates that can take part in chain reactions leading to further oxidations. Not only is the bilirubin molecule lipid soluble and therefore likely to be available for oxidative attack by the lipid peroxidation process in the microsomal membrane, but it also has an easily removable hydrogen atom at the C10 position which enables it to behave in a similar manner to chain breaking antioxidants (Chapter 5). Bilirubin, therefore, could interrupt lipid peroxidation and in turn become oxidised to diazo negative products.

1.4 AIM OF RESEARCH

Our objective was to define an alternative pathway for bilirubin metabolism in the presence of defective glucuronidation of bilirubin using the Gunn rat model. Preliminary experiments indicated that bilirubin could be degraded *in vitro* by a hepatic microsomal system under conditions identical to those required for the initiation of lipid peroxidation. The factors controlling lipid peroxidation and bilirubin oxidation were therefore investigated *in vitro*. Subsequently, lipid peroxidation in Gunn rats was modulated by altering either dietary lipid or the administration of antioxidants in order to investigate the association between lipid peroxidation and bilirubin degradation *in vivo* and the possible role that lipid peroxidation might play in the alternative pathway of bilirubin metabolism.

CHAPTER 2

LIPID PEROXIDATION

Lipid peroxidation (LPO) can be broadly defined as a free radical chain reaction dependent on the oxidative deterioration of polyunsaturated lipids i.e lipids that contain more than two carbon-carbon double covalent bonds (C=C). One of the oldest known examples of oxygen-dependent degradation of polyunsaturated lipids is rancidity that occurs when fats and oils are stored.This problem is of particular importance now with the increased popularity of margarines and cooking oils that are high in polyunsaturates.

The reactions which form the basis of lipid peroxidation were worked out in relation to chemical systems, in the 1940s. The relevance of these reactions to biological systems was not, however, appreciated until later (reviewed by Gutteridge & Halliwell, 1990).

Peroxidation can be initiated by, and gives rise to, reactive species or 'free radicals' (see mechanism below). Free radicals have been implicated in a variety of diseases and their relevance in clinical medicine is receiving much attention. The main issue that needs to be resolved is whether free radicals cause disease or whether they are produced in increased amounts as a result of disease and then contribute to further tissue injury or indeed whether both of these events occur.

FREE RADICALS

2.1.1 DEFINITION:

A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. An unpaired electron is one that is alone in an orbital. A single electron occupying an orbital can choose its direction of

spin. Because electrons are more stable when paired together, radicals are generally more reactive than non-radical species, although they have a wide range of reactivity (Halliwell, 1989).

2.2 BIOLOGICAL FUNCTIONS

Free radicals are products of normal cellular metabolism and molecular oxygen is their main source. The microsomal cytochrome P_{450} electron transport system may be the principal generator of oxygen radicals in hepatocytes but other organelles may also be involved.

Free radicals play a vital role in several life sustaining functions. They are required for the metabolism of many endogenous and exogenous lipids and xenobiotics, for cellular respiration, for the production of prostaglandins and leukotrienes from polyunsaturated fatty acids, and for phagocytic destruction and the immune response.

2.2.1 Bile synthesis and detoxification of xenobiotics

Hepatic microsomal oxidation of cholesterol is required for the synthesis of primary bile acids, which play an essential part in the digestion of fats by emulsification and activation of pancreatic lipase. They are also essential for the absorption of neutral fat, long chain fatty acids, cholesterol and fat soluble vitamins (A,D,E and K) from the intestine.

Many xenobiotics are metabolized by oxidation reactions catalyzed by liver microsomal enzymes; molecular oxygen is used for the direct oxidation of the substrate and give rise to free radical intermediates. The resultant microsomal oxidation may be a cleavage reaction for example of O-C bonds and N-C bonds; consequently the microsomal oxidative enzymes have been termed 'mixed-function' oxygenases.

Although hepatocytes are the main cells in which microsomal oxidations are carried out, similar reactions are catalyzed by microsomal fractions of the lung (10 %), kidney (2 %), and small intestine (2 %) in which the percentages in parenthesis refer to enzyme activity compared with that in liver (Halliwell & Gutteridge, 1989). Microsomal oxidative reactions have also been demonstrated in the placenta and skin. In the liver these reactions may sometimes have far-reaching consequences arising from the inactivation of endogenous hormones, drugs and toxic substances which results in the formation of toxic intermediates and/or end-products (Reed, 1987).

2.2.2 Cellular respiration

Cellular respiration occurs in the mitochondria as the result of oxidation of pyruvic acid, fatty acids and certain amino acids. The energy made available from these oxidations is used in the formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate and involves free radical intermediates. The energy stored in ATP is used in a variety of metabolic functions, for example in membrane transport and in muscle contraction.

2.2.3 Prostaglandin and leukotriene synthesis

Prostaglandins and leukotrienes are 18-22 carbon unsaturated, biologically-active fatty acids. They are found in a wide range of tissues, including the brain and other nervous tissue, lung, kidney, adrenals and spleen. Prostaglandins and leukotrienes are synthesized in tissues from the 20-carbon, tetra-unsaturated arachidonic acid as well as from C18:3 and C25:5 or C22:6 polyunsaturated fatty acids which are the hydrolysed components of plasma membrane phospholipids. These enzyme catalyzed oxidations require the formation of free radical endoperoxides as intermediates.

Thromboxanes and prostacyclin are unstable substances formed from the

endoperoxide intermediate in prostaglandin synthesis. These substances have opposing effects in the control of platelet aggregation and thrombus formation. Certain lipid peroxides are potent and selective inhibitors of prostacyclin synthetase thus allowing the thromboxane system to act unopposed, and thereby predisposing to thrombus formation. Drugs that prevent the formation of lipid peroxides would therefore be important in the therapy of thrombosis.

2.2.4 Phagocytosis and immune response

Free radicals serve not only as chemoattractants and activators of phagocytosing cells but are used as part of the chemical attack by these cells on their targets. They are involved in the immune response to the extent that activated macrophages release interleukin I, superoxide anions and certain free radical reaction products such as aldehydes, which alter the structure and therefore the immunogenicity of membrane domains or gamma globulin.

In spite of the fact that free radicals have these life sustaining functions, there is no doubt that excessive free radical activity is potentially deleterious.

2.3 MECHANISMS OF LIPID PEROXIDATION.

Free radical mediated lipid peroxidation of a typical polyunsaturated fatty acid (PUFA) is depicted in Fig 2.1 and can be divided into three stages: initiation, propagation, and termination (Girotti, 1985).

2.3.1 Initiation

Initiation of lipid peroxidation is triggered by abstraction of a weakly bonded allylic hydrogen from a methylene group ($-CH_2-$) by a strong oxidant such as the hydroxyl radical ($OH^{\bullet}$), the perhydroxyl radical ($HO_2^{\bullet}$), a chelated iron-oxygen complex such as ferryl ($Fe^{2+}O$) or perferryl ($Fe^{2+}O_2$) or the triplet excited state of

a photosensitizing dye. The resulting fatty acid alkyl radical ($L^{\bullet}$) is stabilized by resonating to form a conjugated diene.

2.3.2 Propagation

Fig 2.1 shows one of four possible structures of the stabilized alkyl radical. Addition of molecular oxygen produces a conjugated peroxyl radical ($LOO^{\bullet}$), which on abstracting a hydrogen from another polyunsaturated fatty acid (LH) produces a hydroperoxide (LOOH), and a new alkyl radical. This propagation, or chain reaction phase of peroxidation, can be repeated many times. Thus an initial triggering event can be greatly amplified provided that sufficient oxygen and unoxidized lipid are available.

2.3.3 Termination

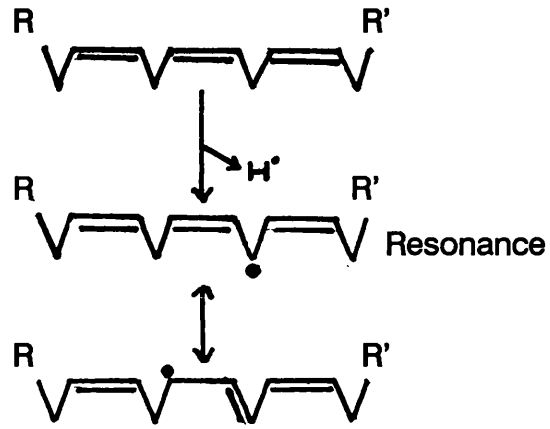
Under normal circumstances, a variety of terminating reactions compete with propagation. Terminations involving two alkyl radicals, two peroxyl radicals, or combinations of these may occur. Other terminations include reactions of alkyl or peroxyl radicals with non-fatty acid substrates (XH) such as phenolic antioxidants eg. α-tocopherol (vitamin E), ascorbate, glutathione or amino acid residues.

2.4 METAL MEDIATED LIPID PEROXIDATION

Transition metals such as iron and copper play a crucial role in the initiation of many lipid peroxidation reactions that proceed by a free radical route. In most cases, these metals in their chelated forms are first activated by reducing agents such as ascorbate, superoxide anion or thiols. LPO can be initiated by metals as described below.

FIG. 2.1
MECHANISM OF LIPID PEROXIDATION

INITIATION:

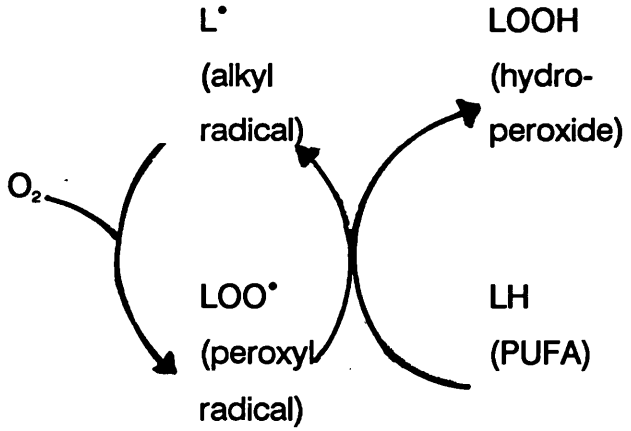


Polyunsaturated Fatty Acid (LH)

Fatty acid alkyl radical ($L^•$)

Stabilized diene ($L^•$)

PROPAGATION:



TERMINATION:

$2L^• \text{ -----> } LL$

$2LOO^• \text{ -----> } LOOL + O_2$

$L^• + LOO^• \text{ -----> } LOOL$

$XH + L^• \text{ -----> } X^• + LH$

$XH + LOO^• \text{ -----> } X^• + LOOH$

(X = Non-fatty acid substrate)

2.4.1 Haber-Weiss cycle.

Here, the initiation of LPO is driven by $O_2^{\bullet -}$ (superoxide anion) and hydrogen peroxide derived from an enzymatic source, for example modified xanthine oxidase acting on xanthine. Iron is generally the redox metal in the Haber-Weiss cycle. This involves coupling of the reduction of Fe^{3+} by $O_2^{\bullet -}$ followed by the reoxidation of Fe^{2+} by H_2O_2, to generate the hydroxyl radical (Fenton reaction).

$$O_2^{\bullet -} + Fe^{3+} \text{ ----> } O_2 + Fe^{2+} \qquad \text{---------- (1)}$$

$$H_2O_2 + Fe^{2+} \text{ ----> } OH^- + \mathbf{OH^\bullet} + Fe^{3+} \qquad \text{---------- (2)}$$

Supporting evidence for the involvement of hydroxyl radical ($OH^\bullet$) in lipid peroxidation initiation in model systems, includes inhibition of LPO by:

(a) $OH^\bullet$ traps such as ethanol.

(b) Superoxide dismutase (SOD) and catalase which catalyse the dismutation of $O_2^{\bullet -}$ and the reduction of H_2O_2 respectivly.

(c) Iron chelators such as desferrioxamine, which supress the reduction of Fe^{3+}.

2.4.2 Iron oxygen complexes

In some cases of iron stimulated lipid peroxidation, iron-oxygen complexes are postulated to be the initiators. A well studied example is the NADPH-cytochrome P_{450} reductase mediated reaction in liver microsomes incubated aerobically with NADPH and ADP-Fe^{3+}. This reaction has been shown to be insensitive to catalase or various hydroxyl radical scavengers, suggesting that an oxidant other than the

hydroxyl radical is involved (Minotti & Aust, 1987).

The possible initiator given most attention is the perferryl ion, which represents a complex between Fe^{2+} and oxygen or Fe^{3+} and superoxide anion (Pedersen et al, 1973).

$$Fe^{2+} + O_2 \text{ ----> } [\ Fe^{II} O_2\ ]^{2+} \text{ <-----> } [\ Fe^{III} O_2^{\bullet -}\ ]^{3+}$$

It has been postulated that adenosine diphosphate forms more stable perferryl complexes than other chelated forms of iron such as EDTA-iron, which may account for the fact that microsomal peroxidation does not occur when EDTA is substituted for ADP (Tien et al, 1982).

2.4.3 Interaction of xenobiotics with chelated metal ions (Reed, 1987).

Xenobiotics (eg. paraquat) can be metabolized to free-radical intermediates which on autooxidation give rise to superoxide anions and then hydrogen peroxide. These intermediates may also reduce chelated metal ions to form hydroxyl radicals. Therefore lipid peroxidation could easily be triggered by xenobiotics especially in microsomal membranes, where they undergo flavoprotein catalyzed electron reduction.

2.4.4 Involvement of metals in lipid hydroperoxide initiated lipid peroxidation.

If significant levels of lipid hydroperoxides (LOOH) pre-exist in cell membranes or polyunsaturated fatty acid preparations exposed to oxidizing conditions, decomposition of LOOH may play a dominant role in the initiation of lipid peroxidation.

LOOH dependent initiation is strongly stimulated by redox metals; the most biologically relevant reactions are iron-catalyzed. There are two general pathways

for LOOH activation:

(a) Oxidation of LOOH by Fe^{3+} and subsequent abstraction of hydrogen by $LOO^{\cdot}$

$$LOOH + Fe^{3+} \text{----> } LOO^{\bullet} + H^{+} + Fe^{2+}$$

$$LOO^{\bullet} + LH \text{ ----> } LOOH + L^{\bullet}$$

(b) Reduction of LOOH by Fe^{2+} and subsequent hydrogen abstraction by the lipoxyl radical, $LO^{\bullet}$

$$LOOH + Fe^{2+} \text{----> } LO^{\bullet} + OH^{-} + Fe^{3+}$$

$$LO^{\bullet} + LH \text{ ----> } LOH + L^{\bullet}$$

Generally the reductive pathway is favoured. It has been shown that iron is also essential for superoxide mediated reduction of LOOH.

2.5 CONSEQUENCES OF LIPID PEROXIDATION

The membranes surrounding cells and cell organelles contain large amounts of polyunsaturated fatty acids. Excessive oxidation of lipids in biological systems can have profound consequences. Organisms normally protect themselves against oxygen damage by a variety of enzymatic and non-enzymatic mechanisms. However, in diseased states, there can be either a breakdown in these defence mechanisms and/or excessive oxidative degradation of membrane lipids or other molecules that contain many C=C bonds.

Lipid peroxidation can cause progressive degeneration of membrane structure and loss of function. Targets for oxidative attack include cholesterol and phospholipids

containing poly-unsaturated fatty acids. Membrane lipid peroxidation may be the underlying cause of many forms of oxygen toxicity, some of which will be discussed. Considered at a molecular level, these cytotoxic effects include:

(a) Structural derangement of the bilayer, decreased membrane fluidity and loss of activity of membrane receptors. Continued fragmentation of fatty acid side chains to produce aldehydes and hydrocarbons such as pentane will eventually lead to complete loss of membrane integrity.
(b) Increased membrane permeability to cytosolic constituents that do not normally cross the membrane such as Ca^{2+} ions, ascorbate and glutathione.
(c) Release of lysosomal enzymes, due to rupture of the lysosomal membrane, into the cell causing increased damage.
(d) Inactivation of intrinsic enzymes and transporters eg. Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase, cytochrome oxidase, and cytochrome P_{450}.
(e) Covalent cross-linking of lipids and proteins.
(f) Polypeptide strand scission.
(g) DNA damage and mutagenesis.
(h) Depletion of NADPH due to antioxidant activity of the glutathione peroxidase/glutathione reductase system.

It is generally considered that many clinical conditions are linked to free radical disturbances (Lunec, 1990). These include: toxic liver injuries (eg. CCl_4, paracetamol), radiation damage, nutritional disorders (eg.antioxidant deficiencies), alcoholism, inflammatory disturbances (eg. arthritis), atherosclerosis, reperfusion injuries (eg. transplantation, tissue grafting, organ storage), cataract, diabetes, some CNS disturbances, cancer, and ageing.

In most of these cases the role, if any, of the free radical disturbances to the clinical disease has not been studied in depth and the connection is often tenuous. Free radicals and/or products of free radical reactions have been detected in damaged or diseased tissues; however this is not sufficient to prove that damage

or disease has been initiated by a free radical reaction. It is known that tissue damage can itself result in release of polyunsaturated fatty acids from phospholipids and perhaps in the release of transition metal ions from bound forms. These events may themselves lead to free radical production so that it is the damage that has stimulated free radical production rather than vice-versa. Thus, although free radical disturbance may be the cause of damage in certain cases (eg. CCl_4 intoxication), they may be a consequence of damage in others.

2.6 FREE RADICALS IN LIVER DISEASE.

Liver disease is a subject of great complexity; the following discussion is limited to some examples of chronic liver disease with which free radicals have been associated (Poli et al, 1987, Braganza, 1989).

2.6.1 Wilson's disease

Wilson's disease is an inherited metabolic defect characterized by low concentrations of caeruloplasmin in the blood and in the progressive accumulation of copper in the liver, kidney, cornea, and brain. The content of copper in normal human livers is 9-47 ug/g dry weight, whereas in patients with Wilson's disease the content is much higher being in the range of 94-1360 ug/g dry weight (Slater, 1989).

The liver in Wilson's disease shows a broad spectrum of abnormal histology ranging from periportal fibrosis, necrosis, to a full-blown cirrhosis. Damage to the brain leads to lack of co-ordination, tremors,and progressive mental retardation. The disease is progressive and fatal if untreated.

It is postulated that copper-stimulated free radical reactions are involved in the pathology of Wilson's disease, although this has not been experimentally proven. Treatment involves a copper restricted diet and use of chelating agents such as

penicillamine that promote copper excretion. Oral administration of zinc salts may also help to prevent copper accumulation, by interfering with the intestinal absorption of copper. Zinc might also compete with copper ions for binding to target sites that could be damaged by free radicals.

2.6.2 Drug-induced liver disease.

2.6.2.1 Carbon tetrachloride (CCl_4).

Carbon tetrachloride, is a good example of a toxic substance that has been much studied in a variety of species. Its lipid solubility allows it to cross cell membranes rapidly, so that it is quickly distributed to all organs; its main toxic effects are, however, shown in the liver. A single dose in the rat produces fat accumulation in the liver due to decreased synthesis of the very low density lipoproteins that transport triglyceride away from this organ. The liver cell endoplasmic reticulum is distorted, hepatic protein synthesis slows down and the activity of other enzymes in the endoplasmic reticulum such as glucose-6-phosphatase and the P_{450} system decline, as does the ability of the reticulum to bind Ca^{2+} ions. The nuclear membrane is attacked more slowly. Eventually there is necrosis of liver cells in the central area of the organ. It has been concluded that the effects produced by CCl_4 are due to the fact that it is a substrate for the cytochrome P_{450} system which is concentrated in the liver. Microsomes from CCl_4-treated rats show increased peroxidation while antioxidants decrease CCl_4 toxicity in animals.

Carbon tetrachloride is thought to produce the carbon-centered radical, trichloromethane ($^{\bullet}CCl3$). The $^{\bullet}CCl3$ radicals can inactivate macromolecules (eg cytochrome P_{450}) by directly binding to them, or they can initiate lipid peroxidation either directly or via another radical ($CCl_3O_2^{\bullet}$, trichloro methylperoxy radical) formed by the reaction with oxygen. It is possible that the local oxygen concentration in different parts of the liver influences whether $^{\bullet}CCl_3$ itself binds covalently to biological molecules or combines with O_2 to form $CCl_3O_2^{\bullet}$ which

would be a better initiator of lipid peroxidation.

The damage caused by CCl_4 may also cause rises in intracellular Ca^{2+} and depletion of glutathione as well as the release of iron ions, thus worsening the damage.

2.6.2.2 Ethanol.

Ethanol is a hepatotoxin that is voluntarily consumed by many of the human population in large quantities in commercial beverages such as beer, wine, or spirits. It is very soluble in both water and in organic solvents, and can cross cell membranes readily. Ethanol absorbed into the body is mainly metabolized in the liver by an enzyme alcohol dehydrogenase to form the aldehyde ethanal. Prolonged intake of excessive amounts of ethanol causes severe damage to many tissues in humans, especially the liver which may become cirrhotic. Hepatic iron overload is common in patients with alcoholic liver diseases and the high incidence of haemochromatosis in different parts of the world has been correlated with the high iron content in local alcoholic beverages.

Chronic exposure of rats to ethanol has been shown to increase hepatic microsomal lipid peroxidation. Large doses of ethanol have also been observed to cause a significant decrease in the glutathione concentrations of liver and kidney cells of rats. Such a drop in glutathione might either cause more lipid peroxidation or merely be a consequence of it. Although the majority of the evidence favours a decrease in gluthathione concentration after chronic ethanol feeding, Kawase et al, (1989) have shown an increase in the hepatic content in rats that are also receiving a low vitamin E diet. Enhancement of lipid peroxidation would activate both enzymatic and non-enzymatic antioxidant protective mechanisms. These defence systems are interrelated and gluthathione levels may first become elevated and then depleted as lipid peroxidation proceeds. The role of lipid peroxidation in ethanol damage to the liver is a subject of debate,

particularly since increased peroxidation often accompanies damage caused by other means.

There is some evidence that damage is caused by ethanal rather than ethanol itself (Baraona et al, 1977, Barry & McGivan, 1985). Ethanal is metabolized mainly by an aldehyde dehydrogenase which converts it to ethanoic acid, but it is possible that some of it could be metabolised by the hepatic enzyme aldehyde oxidase so that, superoxide anions are produced. Ethanal may also react chemically with glutathione and so decrease its concentration.

Analysis of the mechanism of tissue damage by ethanol in chronic alcoholics is made more difficult by the fact that their diet is often inadequate and may lack vitamin E, selenium, thiamine or polyunsaturated fatty acids.

2.6.2.3 Paracetamol.

Paracetamol is a mild pain-killer which in high doses is acutely toxic to the liver and kidneys. It is a substrate for the cytochrome P_{450} system which metabolises it to a highly-reactive species that can attack cell membrane proteins and other proteins by combining with -SH groups. It also causes rapid depletion of glutathione (GSH) so that protection against the hepatotoxicity of paracetamol is provided by giving sulphydryl-containing compounds. These may act by maintaining intracellular GSH concentrations. There is no good evidence that lipid peroxidation causes paracetamol hepatotoxicity. Instead, the GSH depletion and tissue injury caused by paracetamol may lead to increased rates of lipid peroxidation.

2.6.3 Cancer.

Many chemicals are known to produce liver cancer in experimental animals. In humans, high correlations exist between infection with viral hepatitis B or ingestion

of the fungal metabolite aflatoxin B and liver cancer.

An association between cancer and lipid peroxidation was proposed at least twenty years ago. Most of the early work was concentrated on liver tumours where a comparision with normal tissue could be made and low lipid peroxidation activity has been demonstrated (Cheeseman et al, 1989). It has been established that the decrease of lipid peroxidation in hepatomas is directly proportional to the extent to which the cell is differentiated. Thus in hepatomas such as the ascites tumour Yoshida AH-130, there is practically no lipid peroxidation while minimally-deviated hepatomas such as the Morris 44 solid hepatoma show high rates of lipid peroxidation (Dianzani, 1989).

The resistance to lipid peroxidation in tumour tissue could be due to:

(i) A major decrease in the peroxidizable substrate i.e arachidonic acid and other polyunsaturated fatty acids in tumour cell membranes.
(ii) Loss in efficiency of the enzyme activity in the endoplasmic reticulum (NADPH-cytochrome P_{450} system).
(iii) Increased cholesterol in membranes which decreases membrane fluidity and the content of phospholipid unsaturated fatty acids.
(iv) The presence in hepatoma cells of higher amounts of antioxidants such as vitamin E which has been found to be markedly increased in a number of tumours studied by Cheeseman et al, (1989).

Despite these findings, the actual contribution of a single mechanism which increases the resistance to lipid peroxidation still needs to be established. Using models of chemically induced carcinogenesis, it has been established that the decline in lipid peroxidation starts early in the carcinogenic process. Consistant with the finding that the activity of the smooth endoplasmic reticulum is decreased, significant changes in membrane lipid and steroid composition were observed in the hyperplastic nodules. An increase in vitamin E content has been found to

coincide with replicative events (Cheeseman et al 1986). Lipid peroxidation or its products have been suggested to modulate cell growth and proliferation, although experiments are required to confirm this.

2.6.4 Nutritional disorders.

Certain nutritional disturbances, including excessive intakes of vitamin A, low protein diets and deficiencies of antioxidants can result in liver damage. Kwashiorkor, which is an important disease of children especially in the Third World, is associated with protein deficiency but disturbances of liver antioxidants have also been suggested.

Deficiency of antioxidants or excesses of nutrients associated with free radical production result in diseased states, for example when rapid hepatic removal of iron from the plasma results in the liver becoming overloaded. In haemochromatosis the pathology consequent upon iron overload includes liver damage, sometimes leading to hepatoma, weakness and malaise, weight loss, skin pigmentation, diabetes (since pancreatic β-cells are damaged) and cardiac malfunction.

2.7 ANTIOXIDANT DEFENCES

An antioxidant may be defined as 'any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or inhibits oxidation of that substrate' (Halliwell & Gutteridge, 1989). Antioxidants have a vital role to play in preventing or stopping damage due to lipid peroxidation (Niki, 1987). The lipid peroxidation chain reaction, can be terminated by any of the following mechanisms:

(i) Decreases in localized oxygen concentrations (eg. sealing foodstuffs under nitrogen).

(ii) Prevention of initiation by scavenging radicals such as $OH^{\cdot}$.
(iii) Binding of metal ions in forms that will not generate initiating species such as hydroxyl, ferryl or perferryl radicals or decompose lipid peroxides to peroxyl or alkoxyl radicals.
(iv) Conversion of decomposing peroxides to non-radical products such as alcohols.
(v) Chain breaking antioxidants which scavange intermediate radicals such as peroxyl and alkoxyl radicals to prevent continued hydrogen abstraction.

Besides these non-enzymatic antioxidant protection mechanisms, aerobic organisms also have an array of enzymatic defences against oxidative damage (eg. superoxide dismutase, catalase and glutathione peroxidase).

2.7.1 Alpha tocopherol (Vitamin E).

Alpha-tocopherol is the most important lipid-soluble chain breaking antioxidant *in vivo* in humans. Cell membranes and low density lipoprotein (LDL) circulating in the plasma, contain α-tocopherol, which is embedded in the biological membrane or LDL particle. Attached to the hydrophobic structure of α-tocopherol is a hydroxyl group whose hydrogen atom is easily removed. Thus, when peroxyl and alkoxyl radicals are generated during lipid peroxidation, they combine preferentially with the antioxidant instead of an adjacent fatty acid. The antioxidant therefore terminates the chain reaction and α-tocopherol is converted to an α-tocopherol radical, which is poorly reactive and cannot attack adjacent fatty acid side chains. There is considerable evidence that the α-tocopherol radical can be converted back to α-tocopherol by the water soluble antioxidant ascorbic acid or vitamin C (McCay, 1985, Niki, 1987a). Thus, both vitamin C and α-tocopherol contribute to mininizing the consequences of lipid peroxidation in membranes and in LDL.

It has recently been suggested that other compounds may function as antioxidants *in vivo*; these include uric acid (Frei et al, 1988), β-carotene (Burton & Ingold,

1984) and bilirubin (Stocker et al, 1987). However, their physiological importance needs to be established.

2.8 PATHOLOGICAL SIGNIFICANCE OF LIPID PEROXIDATION.

Lipid peroxidation in animal tissues has been shown to increase in various disease states and as the result of poisoning by several toxins. It is generally assumed that the increased lipid peroxidation is then responsible for the subsequent toxicity. This is probably valid in some cases, such as carbon tetrachloride poisoning. However, damaged tissues show increased peroxidisability due to inactivation or loss of antioxidants and the release of metal ions (especially iron and copper) bound to proteins in storage sites. The metal ions are released as the result of hydrolysis by proteolytic enzymes leaking from damaged lysosomes. This sequence of events could explain the elevated lipid peroxidation in disease or toxicity.

In atherosclerosis, rheumatoid arthritis, reoxygenation injury and traumatic or ischaemic damage to the central nervous system, there is evidence to suggest that free radical reactions make a significant contribution to the disease pathology (Lunec, 1990). There is no evidence, however, that increased lipid peroxidation is a major cause of tissue injury in most other diseases; the increased lipid peroxidation may merely accompany the disease state. Even if peroxidation may not be the prime cause of damage, its occurance as a consequence of damage is still biologically important in worsening tissue injury. Therapy with chain breaking antioxidants or with chelating agents that can bind metal ions may therefore be helpful in some diseases.

Oxidant generation can be regarded as a part of normal human metabolism but, when produced in excess, can cause tissue injury. Tissue injury itself can cause additional oxidant generation which may, contribute to further injury. In normal metabolism, a homeostatic balance between lipid peroxidation and antioxidant

protection may occur, so that the mechanism which predominates depends on the animal's disposition at that time. Excessive lipid peroxidation in the disease state whether as a consequence or cause of the disease, depletes the antioxidant protective mechanisms, and continues to do so until these are either replenished or lipid peroxidation is inhibited by some other means.

The use of antioxidants, with accurate methods for the measurement of lipid peroxidation *in vivo*, need to be further investigated in order to evaluate the exact contribution of oxidants to pathology.

CHAPTER 3

IN VITRO ASSAY FOR HEPATIC BILIRUBIN DEGRADATION

3.1 INTRODUCTION

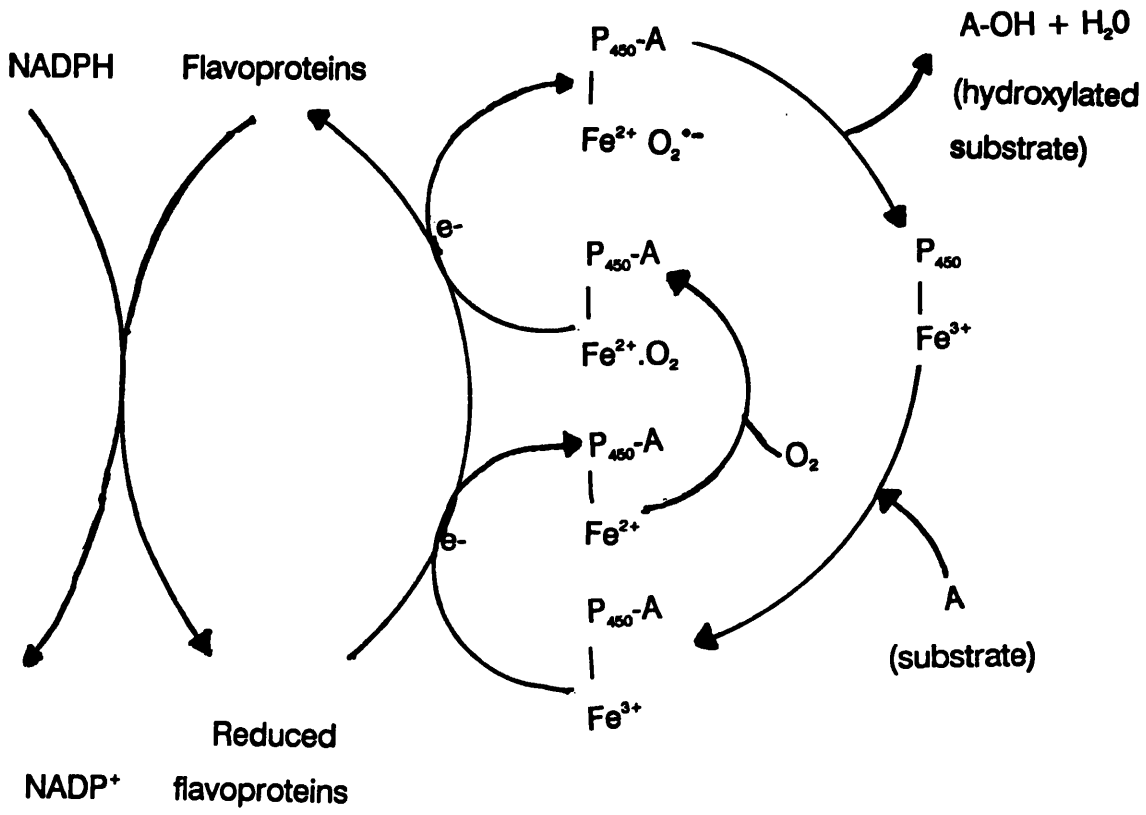
In order to get a better understanding of the mechanism(s) responsible for the elimination of bilirubin in the Gunn rat, as discussed in Chapter 1, the primary objective was to derive an *in vitro* assay for hepatic bilirubin degradation. Hepatic microsomal systems were investigated for the following reasons:

(i) Kapitulnik and Ostrow (1977) had found that administration of a potent inducer of microsomal mixed-function monoxygenases, 2,3,7,8-tetrachlorodibenzo-p-dioxin to Gunn rats produced a significant decrease in plasma BR. They interpreted this finding as indicating that stimulation of alternative pathways of bilirubin metabolism was a microsomal cytochrome P_{450} dependent process.

(ii) Support for the role of the cytochrome P_{450c} isoenzyme (cytochrome P_{448}) came from the further observation that jaundiced Gunn rats, particularly the newborn, had increased microsomal levels of this specific isoenzyme (Kapitulnik et al, 1987).

It is known that the endoplasmic reticulum contains membrane-bound electron transport systems which participate in oxidation reactions. One such system consists of a flavo protein and NADPH-cytochrome P_{450} reductase which feeds electrons to a number of cytochrome P_{450} isoforms (Astrom & DePierre, 1985); its mode of action is illustrated in Fig 3.1. This system is involved in the metabolism of endogenous substrates such as steroids and fatty acids as well as numerous drugs (eg phenobarbitone, morphine, amphetamine and carcinogenic hydrocarbons), which often require a specific cytochrome P_{450} isoform. Both the flavoprotein and the different cytochrome P_{450} isoenzymes are induced by drugs which stimulate the proliferation of the smooth endoplasmic reticulum in the liver.

FIG 3.1 HYDROXYLATION OF SUBSTRATE 'A' BY THE MICROSOMAL ELECTRON TRANSPORT SYSTEM.



The substrate "A" first combines with the Fe^{3+} form of P_{450} which is then reduced by one electron from NADPH to the Fe^{2+} form. The latter is then oxygenated and a second electron from NADPH converts the bound oxygen into an $O_2^{\bullet-}$ radical. An internal oxidoreduction ensues, with the formation of the hydroxylated substrate and H_2O, which contain the oxygen atoms introduced as O_2. Free cytochrome P_{450} is regenerated in its Fe^{3+} form.

Most studies have been performed using the microsomal fraction, obtained by ultracentrifugation of liver homogenates, which consists essentially of vesicles of the fragmented endoplasmic reticulum (80 %) and small amounts of vesicles from plasma membranes, golgi apparatus and outer mitochondrial membranes.

3.1.1 Plan of investigation

This Chapter describes the development of an *in vitro* assay for bilirubin degradation (BRD) and establishes the optimal conditions.

Gunn rat liver microsomes were isolated and the sodium salt of bilirubin was synthesised in order to study the oxidation of bilirubin at a physiological pH. The early work was, however, plagued by the instability of sodium bilirubinate in buffer, and therefore additional means of introducing bilirubin to the reaction mixture were investigated.

Preliminary experiments to optimize assay conditions for a microsomal 'bilirubin oxidase' included testing various co-factor requirements, buffers and the effects of pH, temperature, incubation time and substrate and microsomal protein concentrations. Measurable bilirubin degradation was not obtained unless adenosine diphosphate (ADP) chelated iron was included in the incubation medium. As the result of these observations it was postulated that lipid peroxidation might play an important role in bilirubin metabolism and it was therefore assayed in later studies but not those with sodium bilirubinate.

3.2 METHODS

3.2.1 Chemicals

The majority of chemicals were obtained from Sigma, Poole, Dorset. Bilirubin was purchased from British Drug Houses, Poole, Dorset. All solvents were of ANALAR

grade. Gases were purchased from British Oxygen.

3.2.2 Animals

Gunn and Sprague Dawley rats from the Royal Free Hospital colony, weighing 280-380g were maintained on standard laboratory chow unless otherwise stated. A 12h light/dark cycle, was used in all experiments. Wistar rats were purchased from Bantin and Kingman, Hull.

3.2.3 Statistics

All results have been expressed as means ± SEM except where stated and statistical analysis was performed using the unpaired t-test.

3.2.4 Preparation of rat liver microsomes

A modified centrifugation method was used for the rapid isolation of microsomes, based on the interaction of calcium with microsomes, such that aggregation of microsomes occured in the dilute post-mitochondrial supernatant (Kamath & Rubin 1972).

Rats were sacrificed by a swift blow to the head. The liver was quickly excised and washed in ice cold 0.25 M sucrose. A 20 % (w/v) homogenate in 0.25 M sucrose was prepared using a Potter Elvehjem homogenizer by four passes of a tight-fitting motor driven teflon pestle. The homogenate was centrifuged for 10 min at 11,000 g (12,500 rpm in a 8 x 14 ml rotor) in a Prepspin 50 MSE ultracentrifuge. The post mitochondrial supernatant was diluted 1:5 with ice cold 0.0125 M sucrose containing 8 mM calcium chloride (pH 7.5). The solution was stirred for a few seconds and divided into 30 ml aliquots in 50 ml plastic centrifuge tubes. These were centrifuged at 1,000 g (2,400 rpm) and 4°C for 3 min. The microsomal pellets were washed twice with ice cold 0.15 M potassium chloride and stored at -70 °C.

When required, pellets were resuspended in buffer.

α-glucosidase assay (Seymour & Peters, 1977)

The membrane bound enzyme α-glucosidase (EC.3.2.1.21) was used as the marker enzyme for the microsomal fraction. The specific activity of this enzyme in isolated microsomes was compared with that in the whole homogenate and was found to be 3-5 times greater in microsomes.

Microsomal pellets and whole homogenate were appropriately diluted. 0.05 ml of diluted sample was incubated for 30 min at 37 °C with 0.1 ml 2.5 mM 4-methylumbelliferyl-α-D-glucopyranoside in 150 mM potassium phosphate buffer pH 7.5 containing 0.15% Triton X-100. Blanks were prepared without substrate. After 30 min, the reaction was stopped by adding 1 ml 50 mM buffered glycine/sodium hydroxide (pH 10.4) and the liberated 4-methylumbelliferone was measured in a Perkin Elmer Fluorimeter 204-A. The excitation wave-length was 365 nm with a 0.2 mm slit width and the emission wavelength was 445 nm with a 0.2 mm slit width. Calibration was performed with a freshly prepared standard solution of 4-methylumbelliferone.

3.2.5 Determination of microsomal protein

A diluted sample or standard (0.5 ml) containing approximately 40 $\mu g\ ml^{-1}$ protein was assayed using a modified Lowry technique (Schacterele and Pollack, 1973). Bovine serum albumin was used to prepare the standards.

3.2.6 Preparation of bilirubin substrates

3.2.6.1 SODIUM BILIRUBINATE (Cardenas-Vasquez et al, 1986)

I. Procedure for drying ethanol.

2.5 g of clean dry magnesium turnings, 0,25 g of iodine, and 25-35 ml of 99 % alcohol were mixed together in a round bottomed flask. The mixture was warmed until all the iodine disappeared. Heating was continued until all the magnesium was converted to magnesium ethylate. 450 ml absolute ethanol were then added and the mixture refluxed for 30 min. The alcohol was distilled directly into the vessel it was stored in.

II. Preparation of 3.5mM sodium ethoxide.

250 ml of dry ethanol were placed in a flask fitted with a reflux condenser. 20 mg freshly cut sodium were added in small pieces. When all the sodium dissolved the resultant sodium ethoxide solution, was stored in a glass stoppered bottle.

Sodium bilirubinate synthesis.

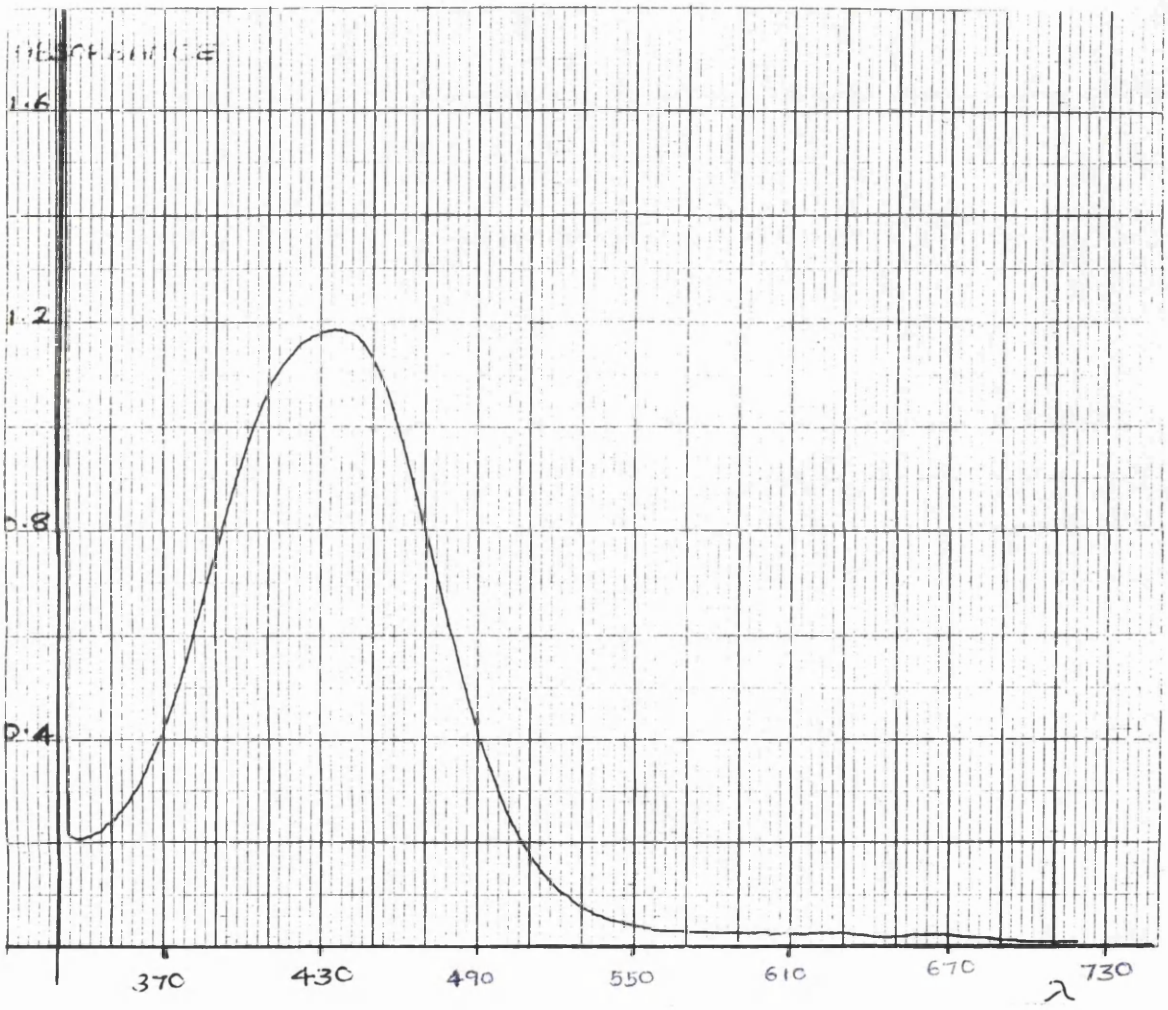
0.257 M solution of bilirubin dissolved in chloroform was made up in the dark. 3.5 mM sodium ethoxide (1 volume) was added to the bilirubin solution (7 volumes), dropwise with mixing. Unreacted bilirubin precipitate was removed by filtration. The azeotropic solution was evapourated using a rotary evapourator and water bath at 50°C. Sodium bilirubinate powder was extracted five times with chloroform to remove any unreacted bilirubin, and once with acetone. The powder was dried under suction and left in a vacuum desicator over self indicating silica gel in the dark and stored under nitrogen at 4 °C.

The absorption spectrum of a solution of sodium bilirubinate in water had one peak at a maximum wavelength of 435 nm (Fig 3.2). The yield of sodium bilirubinate determined on a weight basis was generally 66%. No spectral evidence of biliverdin was detected in the preparations.

3.2.6.2 BILIRUBIN IN DIMETHYLSULPHOXIDE (DMSO) AND SODIUM TAUROCHOLATE

2.5 mg bilirubin were dissolved in 880 µl of DMSO and then added dropwise with

FIG 3.2 ABSORPTION SPECTRUM OF SODIUM BILIRUBINATE.



40 μM Sodium bilirubinate in water, scaned 330-670 nm. A single peak with maximum absorbance at 435 nm was observed.

stirring to approximately 15 ml 40 mM MOPS buffer (3-[N-Morpholino]propane sulphonic acid) pH 7.4, containing 10 mM taurocholic acid. The solution was made up to 20 ml in a volumetric flask (216 μM BR). An appropriate volume of the standard was added to the incubation to give a final BR concentration of 35 μM, DMSO (100 mM) and taurocholate (1.67mM). A fresh solution of bilirubin was prepared for each incubation and used within 2 h.

3.2.7 Determination of bilirubin in incubate

The diazo method of Mertens et al, 1972, was used. The diazo reagent was prepared by mixing 0.15 ml of 10 % (w/v) sodium nitrite with 4 ml of 2 M toluene-4-sulphonic acid; to this mixture 2 ml of a freshly prepared solution of 4-iodoaniline (21 mg ml^{-1}) in glacial acetic acid were added. The mixture was left at room temperature for 2 min, 10 ml of water were added followed by 0.2 ml 1.5 M ammonium sulphamate. The reagent was placed on ice and used between 5 and 20 min after preparation.

(i) 0.5 ml of incubates were pipetted into 14 ml glass-stoppered tubes containing 2 ml acetone:ethanol (1:1, v/v) with 15 mg ml^{-1} butylated hydroxytoluene.
(ii) 0.5 ml diazo reagent was added to each tube. The contents of the tubes were immediately mixed and left for 20 min at room temperature.
(iii) Reaction was stopped by adding 3 ml 1 % (w/v) ascorbic acid in 0.1 M sodium chloride.
(iv) The azo pigment (pink) was extracted into 2 ml butyl acetate, the mixture was centrifuged at 2,600 rpm for 10 min at 4°C. Absorbance of the azo pigment was measured at 530 nm in a Pye Unicam SP6 550 spectrophotometer. Standards of bilirubin were treated similarly. Fig 3.3 shows a standard curve for sodium bilirubinate. Fig 3.4 gives the data obtained when the standard curve was prepared with unconjugated bilirubin in DMSO and taurocholic acid; a regression equation was calculated which was used to estimate the bilirubin concentration in subsequent experiments. Bilirubin degradation in incubation media was followed

by determining the decrease in azo pigment absorption over time (as described below).

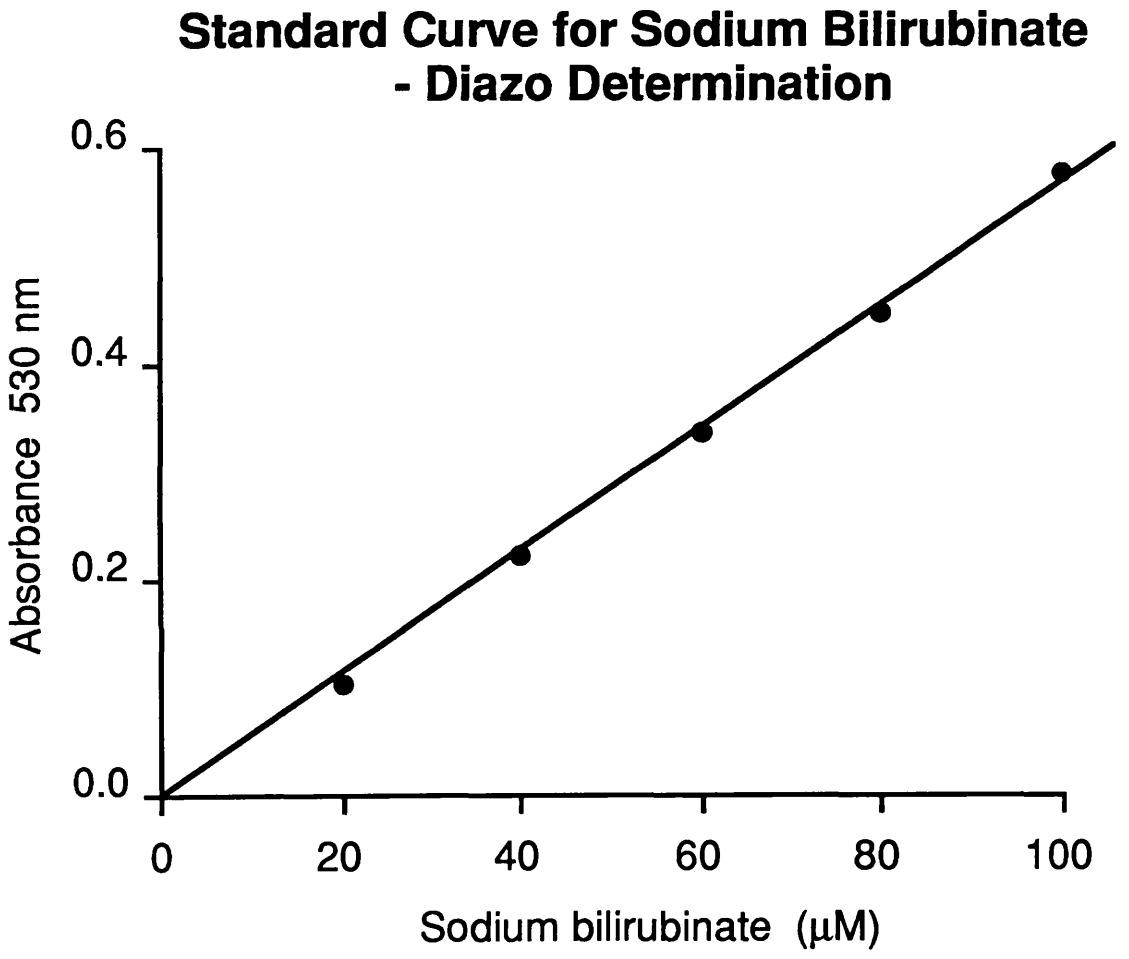
3.2.8 Determination of lipid peroxidation in incubate

- Assay for thiobarbituric acid reactive products.

An adaptation of the method of Yagi by Suenatsu and Abe (1982) was used. Lipid peroxidation activity of microsomes was determined spectroscopically by measuring the rate of formation of thiobarbituric acid reactive products (TBA-RP). Small amounts of malondialdehyde (MDA) are produced during peroxidation and these react in the TBA test to generate a coloured (pink) product. In acid solution the product absorbs light at 532-535 nm. It is readily extracted in solvents such as butanol.

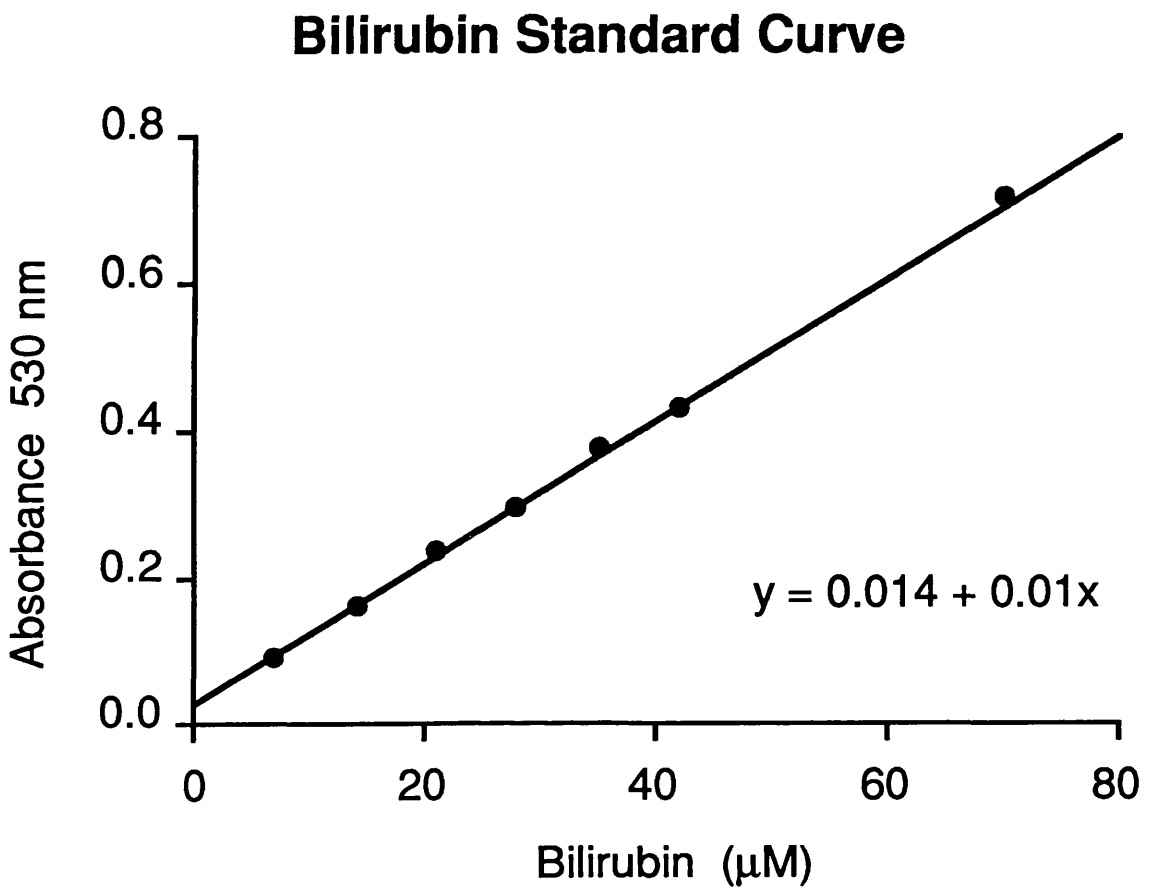
It must be noted that MDA produced during incubation accounts for only a small percentage of the products formed which react with TBA during the assay. Most of the MDA detected by the TBA test is formed by decomposition of lipid peroxides in the presence of iron salts during the acid heating stage of the TBA assay. Artifactual formation of additional lipid peroxidation products from previously unoxidised lipids during colour developmemt was prevented by incorporating butylated hydroxytoluene as an antioxidant in the assay. Formation of MDA has been suggested to be due to the formation of cyclic peroxides and endoperoxides that undergo fragmentation (Gutteridge, 1986). Several compounds, other than MDA, give products that absorb at or close to 532 nm on heating with TBA including, several carbohydrates, amino acids, biliverdin, ethanol and sucrose (Halliwell & Gutteridge, 1989). To account for the contribution of other compounds to TBA reactivity, zero time incubation blanks were always run and lipid peroxidation has been expressed as thiobarbituric acid-reactive products (TBA-RP). However, the production of malondialdehyde is generally accepted as a marker for lipid peroxidation.

Fig 3.3



Sodium bilirubinate (0-100 μM) in 10 mM MOPS buffer, pH 7.4 were diazotised as described in section 3.2.7 and the absorbances of the azo pigments extracted in butyl acetate were determined at 530 nm and plotted against the concentration of sodium bilirubinate.

Fig 3.4



Unconjugated bilirubin was dissolved in DMSO taurocholate as described in section 3.2.6. The absorbance of the azo pigments formed at 530 nm were plotted against the bilirubin concentration. A regression equation of y = 0.014 + 0.01x was obtained.

Reagents:

1. Butylated hydroxytoluene (BHT), 66 mM in methanol.
2. 'Yagi' reagent:
   Sodium dodecyl sulphate, SDS (0.4 g in 5.7 ml H_2O)
   Phosphotungstic acid (0.85 g in 8.5 ml H_2O)
   Thiobarbituric acid (0.19 g in 28.5 ml H_2O)
   57.3 ml 0.1 M HCl added to make up volume to 100ml.
3. n-Butanol.

Assay.

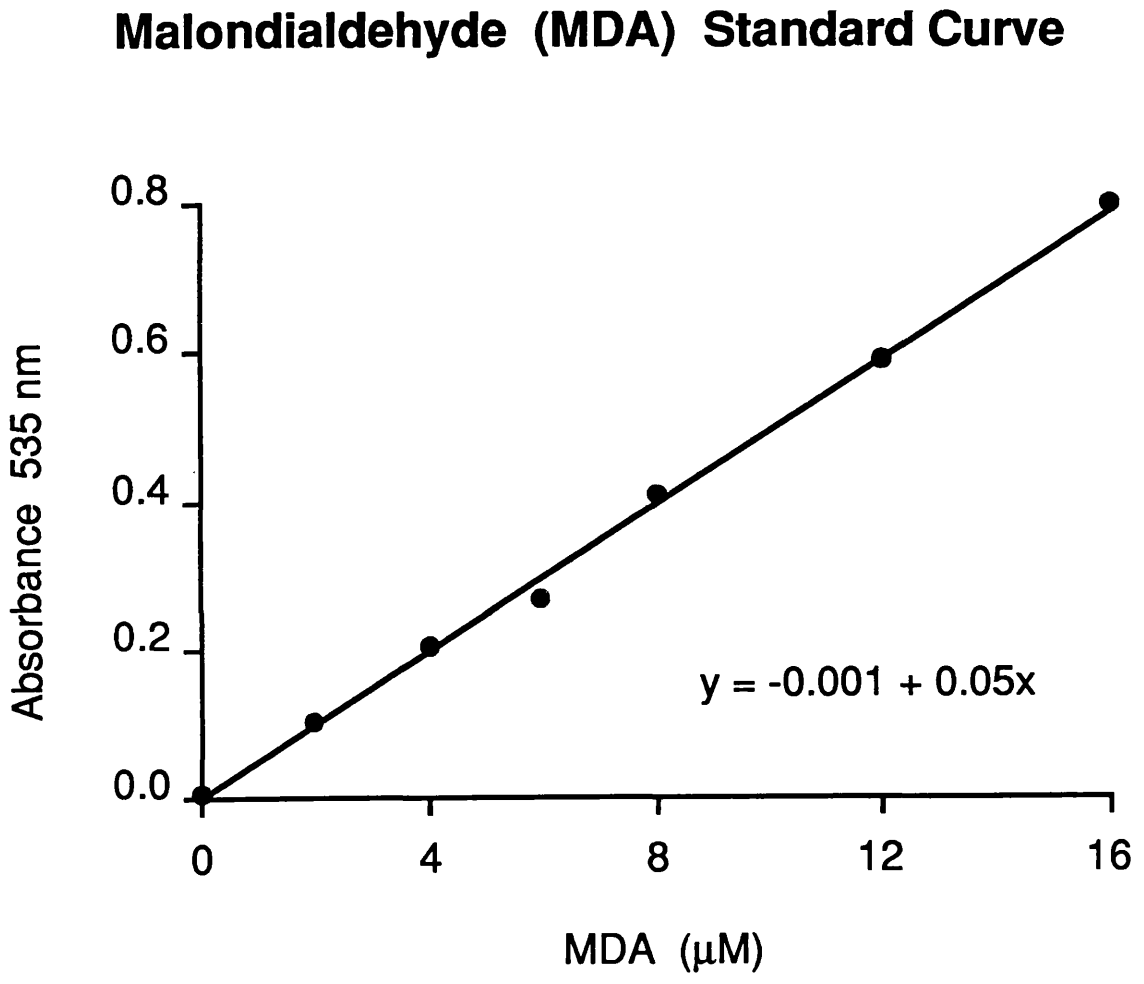
0.5 ml samples of the incubation mixture were added in duplicate, at various time intervals to tubes containing 1.75 ml 'Yagi' reagent, 50 µl BHT in methanol and 0.5 ml water. The tubes were then vortex mixed and heated at 95°C for 60 min in a water bath. After cooling, 2.5 ml butanol were added to each tube, and the contents mixed and centrifuged at 3,000 rpm for 10 min to separate the butanol and aqueous phases. TBA-RP, extracted in butanol, was measured in a Pye Unicam SP6 550 spectrophotometer at 535 nm. The concentration of TBA-RP was determined from an MDA standard curve. LPO was expressed as nmoles of TBA-RP formed per min per mg microsomal protein. Zero time values were routinely measured and subtracted from all TBA-RP assays.

MDA STANDARD CURVE

Construction of a calibration curve for the assay is complicated by the fact that MDA is unstable in aqueous solution and must therefore be prepared immediately before use by hydrolysing a derivative.

Standard solutions of MDA were prepared by adding 100 µl malondialdehyde bis dimethyl acetal, (obtained from Fluka) to 4.9 ml 0.1 M HCl, and incubating at 37°C

Fig 3.5



Malondialdehyde bis dimethylacetal was treated as described in section 3.2.8 to give concentrations of MDA ranging from 0-16 μM in the TBA-RP reaction. When these concentrations of MDA were plotted against the absorbance of the solution at 535 nm a regression equation of y = -0.001 + 0.05x was obtained.

for 15 min to hydrolyse the acetal to yield 0.122 M MDA. This was diluted with buffer (MOPS pH 7.4) to give a stock solution of 32 μM MDA, which was used to prepare standard solutions.

Samples of MDA standards were then assayed for MDA in exactly the same way as the samples of the incubation mixture.

Using these values a regression line was calculated and used to determine MDA concentrations for given absorbances (Fig 3.5)

3.2.9 Incubation conditions

3.1.9.1 Enzymatic

Incubations (8 ml), were carried out in 25 ml Erlenmeyer flasks containing the following final concentrations: 40 mM MOPS buffer pH 7.4, an NADPH generating system (0.18 mM $NADP^+$, 4.03 mM glucose-6-phosphate, 0.19 IU ml^{-1} glucose-6-phosphate dehydrogenase), 1.6 mM ADP, 18 μM ferrous sulphate, 35 μM sodium bilirubinate or unconjugated bilirubin dissolved in DMSO and sodium taurocholate and 1.5-2.0 mg ml^{-1} microsomal protein suspension. All components of the incubation medium were made up in the buffer, except ferrous sulphate which was made up in water.

The reaction was started by the addition of microsomes. Incubations were carried out at 37 °C, aerobically, in a shaking water bath in dim light. Samples were removed in duplicate at various time intervals, to determine both the disappearance of bilirubin, as previously described and the progression of lipid peroxidation. Lipid peroxidation (LPO) and bilirubin degradation (BRD) activities were calculated for 10 min of incubation and expressed as nmol min^{-1} mg $prot^{-1}$.

3.2.9.2 Non enzymatic

The non-enzymatic incubation system was essentially the same as the above enzymatic system, with the exception that the NADPH generating system was replaced with 0.2 mM ascorbic acid.

In some incubations, microsomal enzymes were denatured by incubating the microsomal suspension at 80-83 °C for 5 min prior to addition to incubation flask

3.3 RESULTS AND DISCUSSION

3.3.1 Sodium bilirubinate - preliminary experiments

Sodium bilirubinate was the substrate first selected to investigate hepatic microsomal bilirubin oxidation since it is soluble in aqueous solution at a physiological pH. In these studies, the decrease in bilirubin absorbance proceeded linearly for 10 min and this incubation time was therefore chosen for the calculation of bilirubin degradation activity (Fig 3.6). The rate of disappearance of bilirubin from the incubate, was expressed as the percentage change in BR concentration at 10 min.

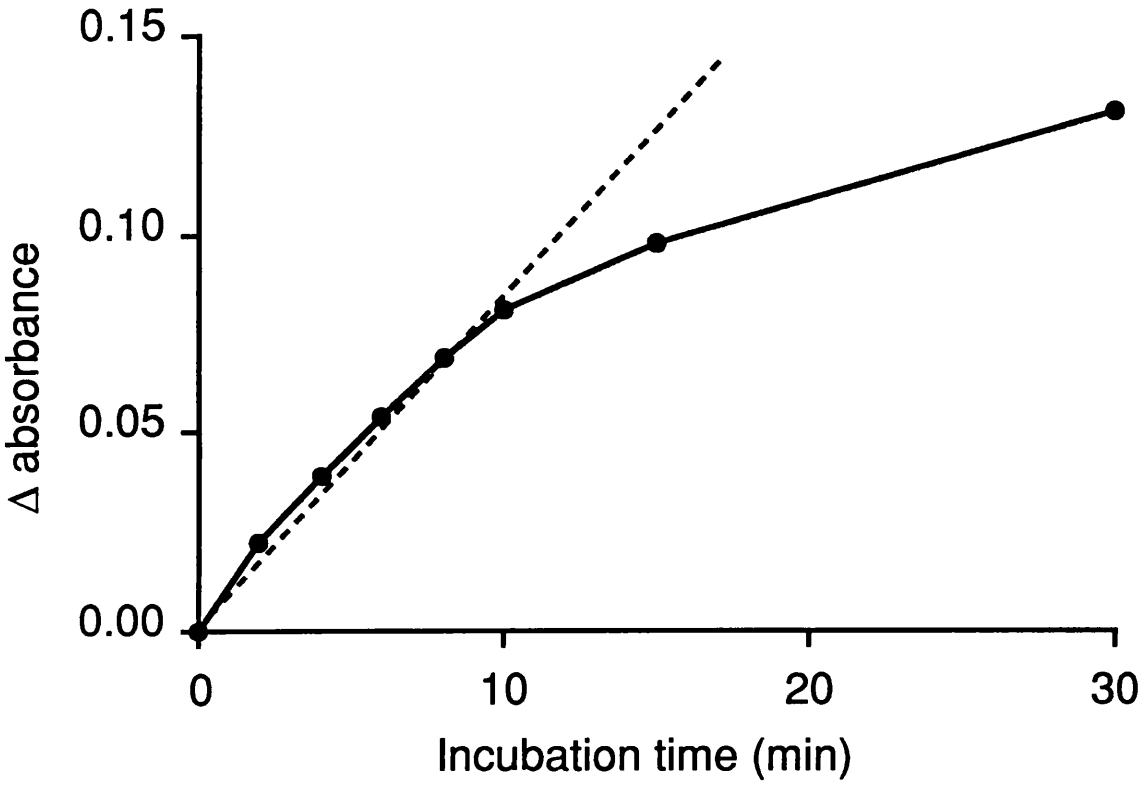
Table 3.1 shows that NADPH, ADP and $FeSO_4$ are essential cofactors in sodium bilirubinate degradation by microsomes in the presence of MOPS buffer (10 mM) pH 7.4.

Fig 3.7 shows that a concentration of 18 μM $FeSO_4$ was optimal and was therefore selected for subsequent studies.

3.3.2 Change of substrate

Having obtained a system in which sodium bilirubinate could be degraded by

Determination of Optimum Incubation Time



The decrease in absorbance of the bilirubin azo pigments (A_o -A_t) at 530nm was plotted against the incubation time (0 - 30 min). Linearity (—) was achieved up to 10 min.

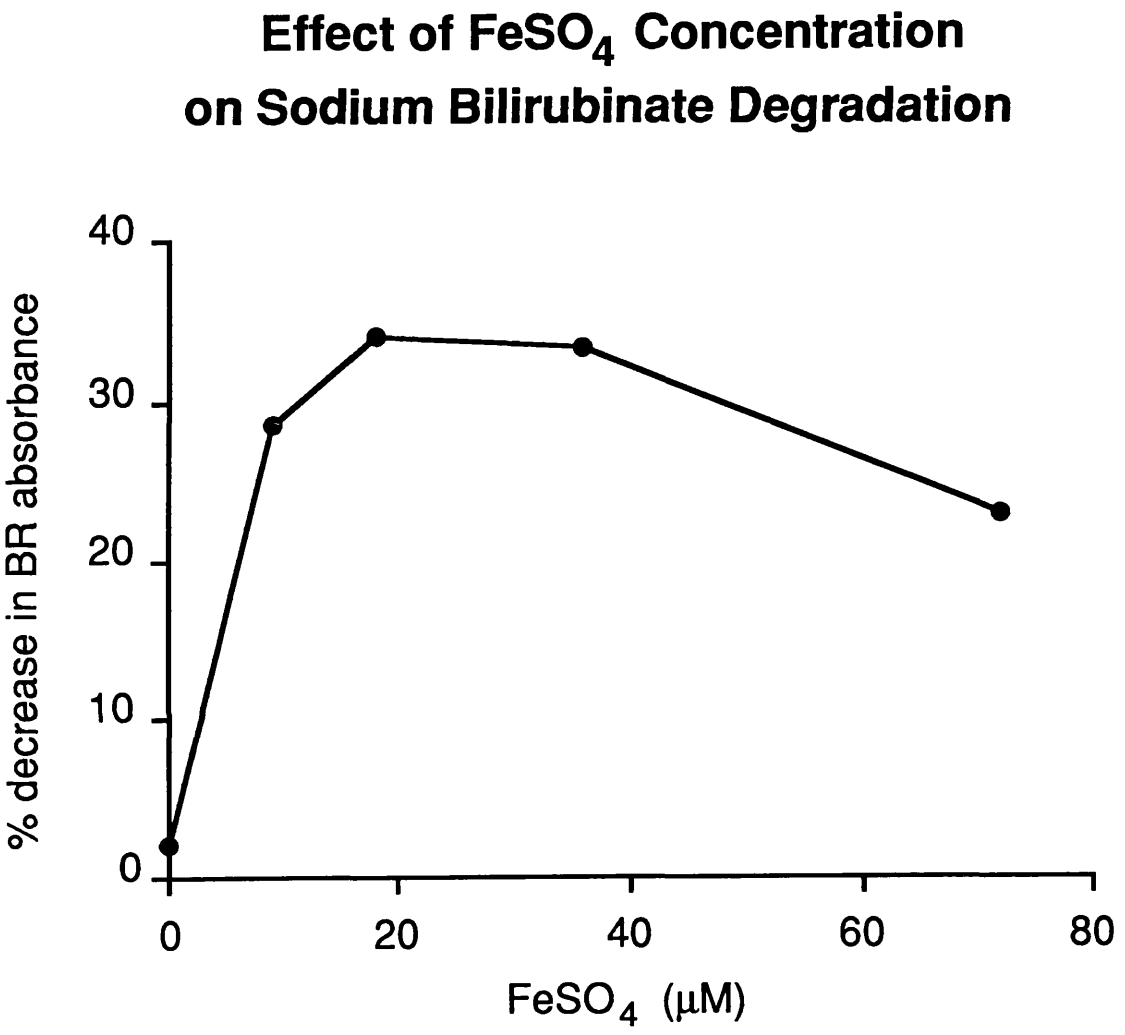
TABLE 3.1 CO-FACTOR REQUIREMENTS FOR SODIUM BILIRUBINATE DEGRADATION

	Mean % decrease in sodium bilirubinate conc.
* Complete system	31
- minus NADPH	5
- minus ADP	10
- minus $FeSO_4$	6

* Complete system: Microsomes (1-2 $mgml^{-1}$), NADPH generating system (0.18 mM $NADP^+$, 4.03 mM G6P, 0.19 IU ml^{-1} G6PD), ADP (1.6 mM), $FeSO_4$ (18 μM), sodium bilirubinate (36 μM), 10 mM MOPS buffer pH 7.4. In experiments with unconjugated BR, 40mM MOPS was used.

Incubations were carried out at 37^0C in a shaking water bath under dim light. Percentage decrease in sodium bilirubinate concentration was calculated at ten minutes.

Fig 3.7



Concentrations of $FeSO_4$ varying from 0 - 70 μM were added to the standard incubation mixture containing 35 μM sodium bilirubinate. Azo-pigment determinations were determined at 0 and 10 min and the difference expressed as percentage change; this value provided an index of the amount of sodium bilirubinate degraded.

microsomes, this substrate had to be abandoned because it became apparent that in the absence of microsomal protein, sodium bilirubinate was highly unstable. Despite using different buffers in the presence and absence of EDTA, a more alkaline pH, and bilirubin bound to albumin, it was not possible to develope a system where both the substrate blank was stable and linear bilirubin degradation was obtained over the incubation period. Moreover, there was the problem of the preparation of sodium bilirubinate which was time consuming and had the disadvantage of lack of a standard with which to compare the degree of purity of the synthesised compound.

Unconjugated bilirubin (BDH), dissolved in dimethyl sulphoxide (DMSO) and solubilized with taurocholic acid was therefore selected as the substrate in all of the following studies. It was found to be stable in the absence of microsomes and could also be degraded under the incubation conditions described.

Sodium bilirubinate gave a value for bilirubin degradation of 0.37 ± 0.06 nmol min^{-1} mg $prot^{-1}$, n = 7 which was comparable to that obtained with BR dissolved in DMSO and taurocholic acid (see below). This suggests that the solvent DMSO/taurocholate does not affect bilirubin degradation activity in the system studied.

3.3.3 Bilirubin degradation *in vitro*

Co-factor requirements

Bilirubin solubilized in DMSO and taurocholic acid was found to have the same co-factor requirements for BRD activity as sodium bilirubinate. Both NADPH and ADP chelated $FeSO_4$ were also found to be essential for LPO activity (Table 3.2).

Effect of microsomal protein

Bilirubin degradation was determined with microsomal protein concentrations in the incubate ranging from 0.5 to 4.3 mg ml^{-1} (Fig 3.8). Incubations were carried out for 15 min under the standard conditions described and changes in BR concentration were expressed as changes in azo pigment absorbance from zero time. The results show that bilirubin degradation increases with increasing protein concentration up to 2.0 mg ml^{-1} but at higher concentrations inhibition of bilirubin degradation occurs. In order to obtain adequate sensitivity for the azo pigment determinations, protein concentrations ranging from 1.5-2.0 mg ml^{-1} were usually employed.

Optimum pH for lipid peroxidation and bilirubin degradation in enzymatic and non-enzymatic systems

To determine the optimum pH for both LPO and BRD in the enzymatically and non-enzymatically initiated systems, incubations were performed at pHs 6.5 7.0, 7.4, and 7.9 (Fig 3.9). Different pH effects were observed for LPO and BRD activities. The optimum pH for BRD was at the physiological pH of 7.4 and was independent of the mode of LPO initiation. This pH was, therefore, selected for the assay.

Oxygen requirement

To demonstrate that lipid peroxidation and bilirubin degradation were aerobic processes, incubations were performed under 100 % N_2, 2 % O_2 and atmospheric oxygen (20%). Buffer was equilibriated at room temperature at the appropriate oxygen concentration by bubbling the gas through it for 90 min before use. Gas was blown over the surface of incubation medium during the assay and samples were removed from a side arm tube via a syringe attached to a three way tap in order to avoid altering the gaseous environment of the incubation. All other

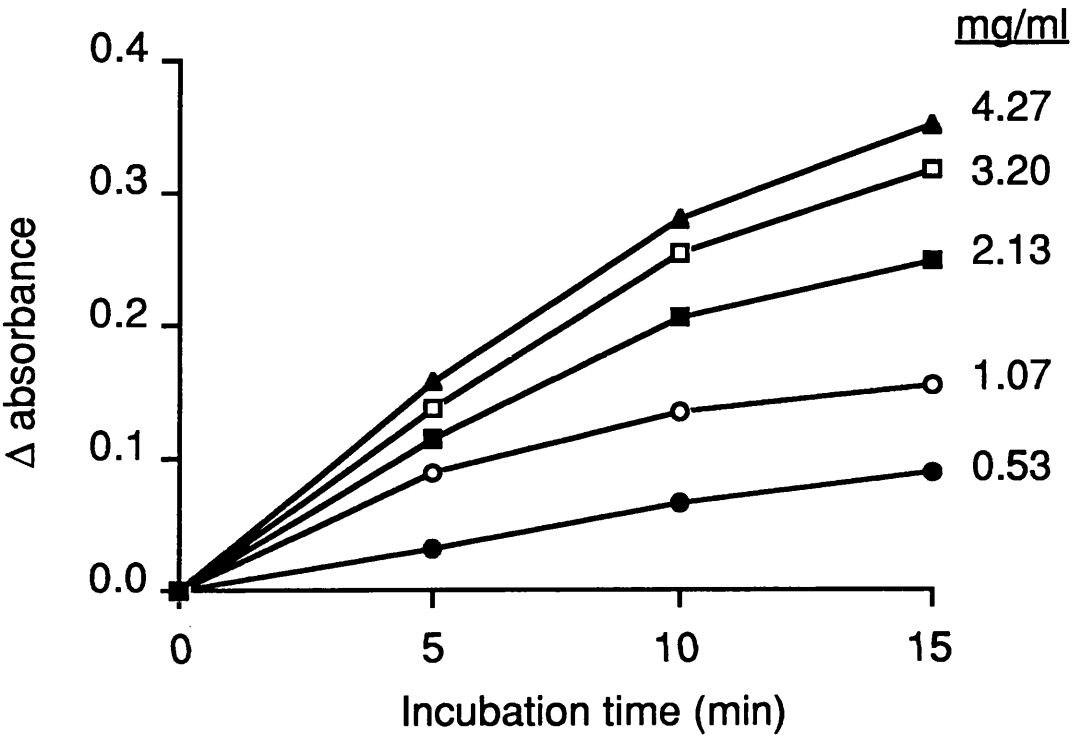
TABLE 3.2 CO-FACTOR REQUIREMENTS FOR LPO AND BRD ACTIVITIES

	n	Mean activity ± SEM (nmol min^{-1} mg $prot^{-1}$) LPO	BRD
* Complete system	23	1.26 ± 0.09	0.40 ± 0.04
- minus NADPH	3	0.02 ± 0.02	0.00
- minus $FeSO_4$, ADP	3	0.00	0.00

* See table 3.1

Fig 3.8

Effect of Microsomal Protein Concentration on Bilirubin Degradation



Incubations were performed with unconjugated bilirubin in DMSO and taurocholic acid (35 μM) and microsomal protein concentrations ranging from 0.53-4.27 mg ml^{-1} as described in section 3.2.9. Aliquots were removed at 0, 5, 10 and 15 min and the decrease in azo pigment absorbance (A_o - A_t) at 530 nm plotted against time to indicate the relative amounts of bilirubin degraded.

incubation conditions were as described above. LPO and BRD were determined at various time intervals (Fig 3.10).

Both LPO and BRD had maximal activity at atmospheric oxygen, lower activities were observed at 2 % oxygen and complete inhibition at 100 % nitrogen (0 % oxygen).

3.3.4 Does bilirubin interefere with the MDA assay ?

Interference in the MDA assay by BR has been suggested as a result of the conversion of bilirubin to biliverdin, (Tickner & Gutteridge, 1978: and Gutteridge & Tickner, 1978) which forms a red-brown complex with thiobarbituric acid.

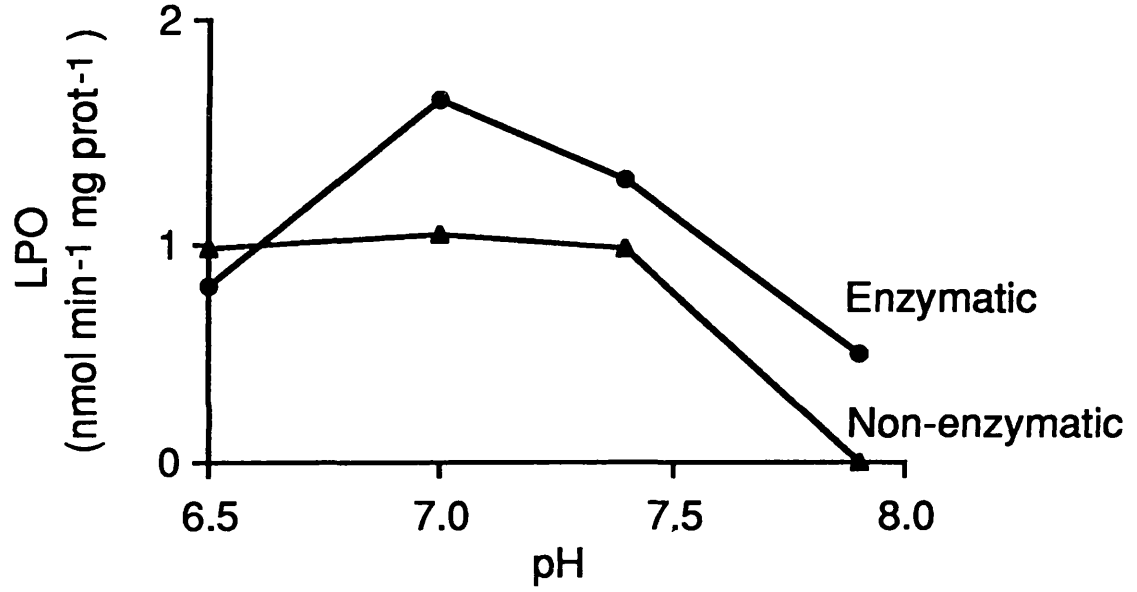
To test this hypothesis, experiments were carried out in which pink butanol extracts of the TBA-RP assay for lipid peroxidation, obtained under standard incubation conditions, were shaken with 10 M NaOH, which is known to turn the biliverdin-TBA complex blue. There was, however, no evidence of formation of the blue biliverdin complex, either visually or by spectral analysis.

Moreover, in the *in vitro* assay there was no direct evidence of biliverdin formation; the yellow bilirubin colour tends to disappear during incubation and formation of the green biliverdin compound has not been observed or detected spectroscopically. It is also of interest to note that biliverdin was not found to be a substrate for degradation by the microsomal system used to degrade bilirubin, even after 30 min incubation.

Since bilirubin absorbs maximally at 430 nm, and TBA-RP absorbs at 535 nm, an overlap in absorbance spectra in the LPO assay was not anticipated. Nevertheless, to test the extent to which bilirubin might interfere in the MDA assay the absorbance of the Yagi derivative of 2-12 μM MDA, was measured in the presence and absence of 35 μM bilirubin. MDA standards were prepared as described

Fig 3.9

Optimum pH for LPO and BRD in Enzymatic and Non-Enzymatic Systems



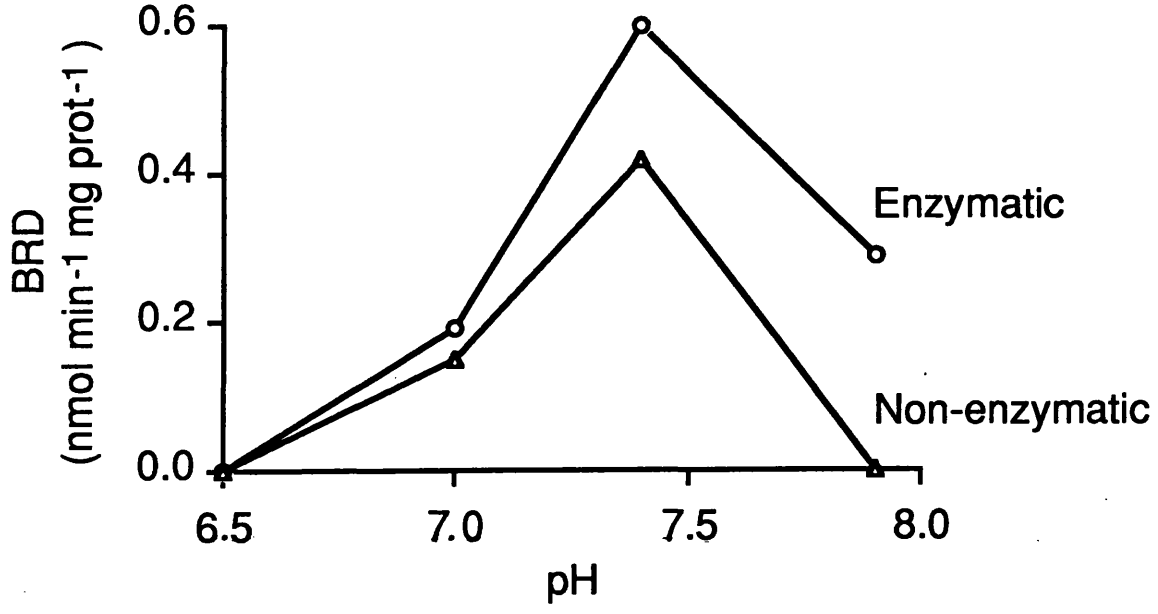
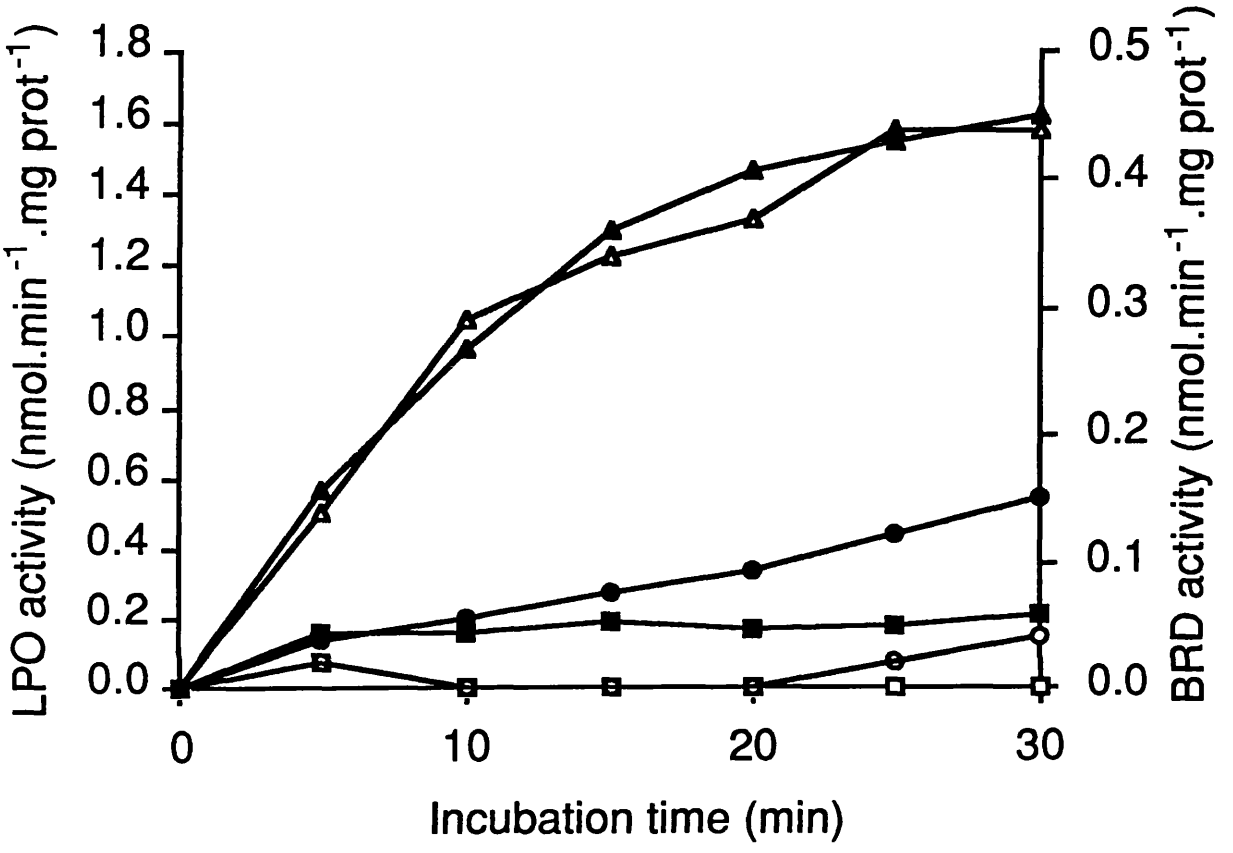


Fig 3.10

Oxygen Dependence of Lipid Peroxidation and Bilirubin Degradation Activities



Closed symbols reprsent LPO,
Open symbols BRD.

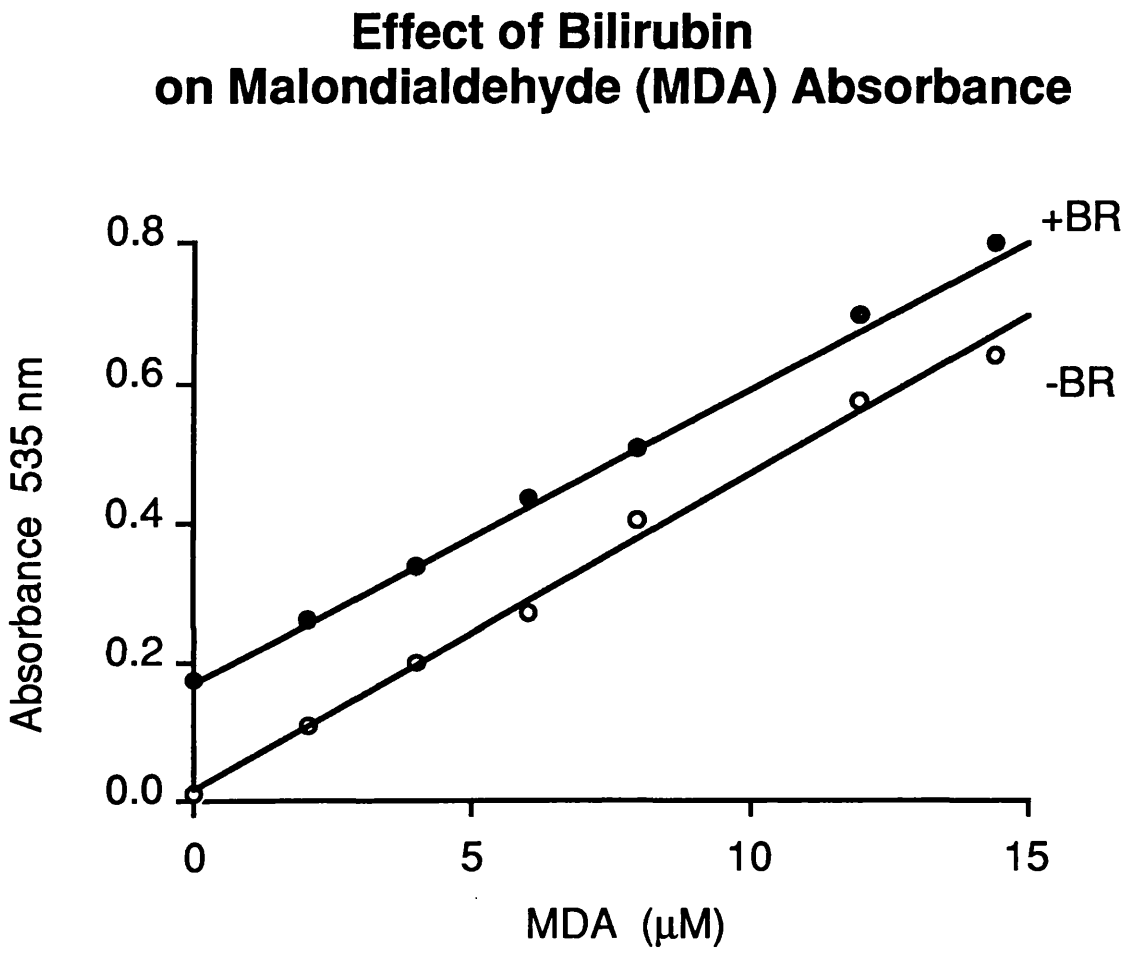
100% N_2 ■ □

2% O_2 ● ○

20% O_2 ▲ △

Standard incubations were performed as described in section 3.2.9 at different oxygen concentrations (0, 2 and 20%). Samples were withdrawn at 0,5,10,15,20,25 and 30 min for determinations of LPO and BRD activities. Closed symbols represent LPO; open symbols BRD.

Fig 3.11



A standard curve for MDA was obtained as previously described (3.2.8) in the presence and absence of bilirubin (35 μM). Bilirubin did not have a significant effect on the slope of the curve.

above under methods. A consistent increase in MDA absorbance was obtained at all concentrations when bilirubin was added to the assay (Fig 3.11). However, this does not present a problem in the calculation of MDA concentration because zero time values were routinely measured and subtracted from all TBA-RP determinations in LPO assays. In 5 experiments these averaged only 16 ± 4% of the total TBA-RP formed in 10 min. In conclusion, our studies provide no support for the hypothesis that the presence of bilirubin in the LPO assay, influences the absorbance of TBA-RP by becoming converted to biliverdin.

3.3.5 Effect of DMSO and sodium taurocholate on lipid peroxidation activity

Dimethyl sulphoxide and sodium taurocholate were used to solubilize bilirubin in the *in vitro* assay.

Bile acids have recently been reported to inhibit lipid peroxidation (De Lange & Glazer, 1990), It therefore became necessary to test the effect of DMSO and taurocholate on lipid peroxidation activity. Hepatic microsomes from 4 Gunn rats were incubated with NADPH, ADP and $FeSO_4$ under standard conditions, in the presence and absence of DMSO and sodium taurocholate. The mean LPO activities were 1.29 ± 0.10 and 1.64 ± 0.11 nmol min^{-1} mg $prot^{-1}$ respectively. This indicates a 21 % inhibition of LPO activity by DMSO/taurocholate. Since almost all of the *in vitro* assays involved a comparision of LPO and BRD activities and since bilirubin did not influence the DMSO/taurocholate inhibition of LPO when added to the assay, (activity = 1.23 ± 0.11 nmol min^{-1} mg $prot^{-1}$), the small inhibitory effect of DMSO / taurocholate was disregarded.

CHAPTER 4

ROLE OF LIPID PEROXIDATION AND BILIRUBIN DEGRADATION IN VITRO.

INTRODUCTION

The *in vitro* assay conditions for LPO and BRD described in Chapter 3, were used in the following studies to determine to what extent the two activities are related.

(i) Hepatic microsomal activities were altered by:

- initiating LPO enzymatically or non-enzymatically,
- subjecting the microsomal enzymes to heat denaturation,
- adding inhibitors of LPO (high conc of ascorbic acid, GSH) to the incubation system.

(ii) The BRD capacity of other oxidative species which may or may not be directly involved in LPO, was assessed.

(iii) The lipid antioxidant properties of bilirubin were examined.

4.1 ENZYMATIC AND NON-ENZYMATICALLY INITIATED LPO:

Enzymatically initiated LPO in the hepatic microsomal system involves the enzymes NADPH-cytochrome P_{450} reductase and possibly cytochrome P_{450} which donates electrons to iron complexes and are therefore responsible for generating Fe^{2+} which stimulates peroxidation (Pederson & Aust, 1975, Svingen et al, 1979). The

function of this enzyme system is similar to that of ascorbic acid in non-enzymatic LPO, where low concentrations of ascorbate, in the presence of ionic iron and oxygen, are thought to stimulate LPO by reducing Fe^{3+} to Fe^{2+}. Ferrous iron forms oxygen radical species which stimulate LPO, via the mechanism described in Chapter 3.

In contrast high concentrations of ascorbate inhibit LPO probably by reducing some of the lipid peroxy radicals directly to hydroperoxides and thus interfering with the chain reaction (Halliwell & Gutteridge 1989). Ascorbate can also regenerate vitamin E, the chain-breaking anitoxidant in biological membranes and thus may also be acting indirectly to inhibit LPO (Niki, 1987).

Glutathione is generally regarded as having a protective role against oxidative toxicity *in vivo* because it is a substrate for glutathione peroxidases and glutathione transferases which destroy hydrogen peroxide and various organic hydroperoxides. It also scavenges hydroxyl radicals and singlet oxygen. GSH can also react directly with various aldehydes produced during LPO and so protects the thiol groups of membrane proteins. The addition of GSH to a microsomal LPO system has also been found to lower the concentration of vitamin E required to inhibit LPO (Leedle & Aust 1990). Treatment of rats or mice with reagents that decrease hepatic GSH concentrations increase the susceptibility of their hepatic lipids to peroxidation (Maellaro et al 1989).

4.2 OTHER POSSIBLE OXIDATIVE SPECIES INVOLVED IN LPO AND BRD

The role of oxidative species other than the iron-oxygen complex, that may be involved in the initiation of LPO in the *in vitro* system described, was examined. Superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) were tested in order to assess their ability to act as oxidants of BR or initiators of LPO.

Microsomal lipids are susceptible to iron-dependent peroxidation, although the

mechanism of the process is not well understood (Aust et al 1985, Girotti, 1985, Halliwell & Gutteridge, 1989). Peroxidation of lipids within membranes is inhibited by EDTA, does not require H_2O_2 and is not inhibited by hydroxyl radical scavengers (Tien et al, 1982, Gutteridge, 1982, Girotti & Thomas, 1984). The chelators ADP and ATP efficiently stimulate microsomal LPO (Aust et al 1985, Hochstein & Ernster, 1964, Tien et al 1982), but not hydroxyl radical formation.

4.2.1 Superoxide radical

The enzyme superoxide dismutase (SOD) is ubiquitous and specifically catalyses removal of the superoxide radical ($O_2^{\cdot -}$).

(i) $2\ O_2^{\cdot -} + 2H \xrightarrow{SOD} H_2O_2 + O_2 + SOD$

(ii) $O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe^{3+}\ Fe^{2+}} OH^- + OH^{\cdot} + O_2$ (Haber-Weiss)

(a) $O_2^{\cdot -} + Fe^{3+} \longrightarrow Fe^{2+} + O_2$

(b) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^-$

This suggests that this radical could be a major factor in oxygen toxicity should it persist. Most oxygen taken up by animals is reduced to form water by the electron transport chains of mitochondria and endoplasmic reticulum. As discussed in Chapter 3, the reducing equivalents required by the cytochrome P_{450} system for the metabolism of a wide range of substrates, are donated by NADPH via the flavoprotein enzyme NADPH-cytochrome P_{450} reductase. The two main components of the cytochrome P_{450} system that are considered as potential sources of superoxide anions are shown in Fig.4.1. (i) NADPH cytochrome P_{450} reductase 'leaks' electrons to O_2 so that it is reduced to form the superoxide radical. (ii) The oxygenated intermediate of cytochrome P_{450} breaks down to release $O_2^{\cdot -}$ in a minor side reaction.

Superoxide anions have insufficient energy to abstract hydrogen from polyunsaturated fatty acids but may be involved in the promotion of peroxidation of lipid extracted from microsomes (Pederson & Aust 1973, 1975), in a reaction involving singlet oxygen and is thought to be generated by the non-enzymatic dismutation of $O_2^{\cdot -}$. However, the ubiquitous presence of SOD which catalyses the dismutation of $O_2^{\cdot -}$, means that singlet oxygen is unlikely to be physiologically significant.

Superoxide radical generating systems can be damaging to biological material. This may be due to direct damage by $O_2^{\cdot -}$, or by $HO_2^{\cdot}$, or H_2O_2 formed by dismutation of $O_2^{\cdot -}$. Other reactive species can be formed by the interaction of $O_2^{\cdot -}$ with metal ions such as an oxidant (eg perferryl) or a reductant followed by reoxidation of the reduced metal ion with H_2O_2 to form the hydroxyl radical. Although $O_2^{\cdot -}$ itself is not reported to damage proteins, it does exacerbate the damage produced by $OH^{\cdot}$.

Production of $O_2^{\cdot -}$ would be likely to occur in all aerobic cells and under the *in vitro* incubation conditions described, but it is very difficult to assess the actual amount produced. The superoxide radical itself is poorly reactive in aqueous solution. The protonated form of $O_2^{\cdot -}$, the hydroperoxyl radical ($HO_2^{\cdot}$), is more reactive than $O_2^{\cdot -}$. Hydroperoxyl radicals can abstract a H from polyunsaturated fatty acids and thus initiate peroxidation. Much of the $O_2^{\cdot -}$ generated within cells comes from membrane bound systems (eg the endoplasmic reticulum and the electron transport chains of mitochondria) and so $HO_2^{\cdot}$ formed close to the membrane could conceivably produce damage. Any $HO_2^{\cdot}$ produced in the hydrophobic membrane interior could be very damaging since it is highly reactive in organic media. However the importance of $O_2^{\cdot -}$ or $HO_2^{\cdot}$ in membrane damage *in vivo* remains to be demonstrated.

It is likely that $O_2^{\cdot -}$ exerts many of its damaging effects by the generation of more reactive species. It seems feasible, therefore, that under the *in vitro* conditions studied, some of these reactive species or $O_2^{\cdot -}$ itself (or $HO_2^{\cdot}$) could initiate lipid peroxidation. Also $O_2^{\cdot -}$ or $HO_2^{\cdot}$ might have a direct effect on BR. Experiments were therefore carried out to determine the role of $O_2^{\cdot -}$ in LPO and BRD in the system

studied.

4.2.2 Hydroxyl radicals

The hydroxyl radical is one of the most reactive chemical species known and reacts with almost every type of molecule found in living cells, for example sugars, amino acids, phospholipids, nucleotides and organic acids.

In studies with lysosomal membranes, Fong et al (1973), suggested that the NADPH-dependent peroxidation of liver subcellular membranes is initiated by the hydroxyl radical. They implied that superoxide anion, generated by flavin oxidation, forms hydrogen peroxide which then reacts with more superoxide to form hydroxyl radicals. The hydroxyl radical would then abstract a hydrogen from a methylene carbon atom of the fatty acids to initiate hydroperoxide formation. It might be possible by this mechanism, for hydroxyl radicals to degrade BR either directly or indirectly by initiating LPO.

The reaction between the superoxide radical and hydrogen peroxide to form the highly-reactive hydroxyl radical, is catalysed by traces of metal ions; iron is likely to be the most important *in vivo*:

$$Fe^{3+}\text{-complex} + O_2^{\cdot -} \text{ -----> } Fe^{2+}\text{-complex} + O_2$$

$$Fe^{2+}\text{-complex} + H_2O_2 \text{ -----> } OH^{\cdot} + OH^{-} + Fe^{3+}\text{-complex}$$

Iron catalyst

Net: $H_2O_2 + O_2^{\cdot -}$ --------> $O_2 + OH^{\cdot} + OH^{-}$

Both $O_2^{\cdot -}$ and H_2O_2 can release iron ions from iron proteins, such as haemoglobin. Thus increased generation of $O_2^{\cdot -}$ and H_2O_2 could create the conditions that lead to $OH^{\cdot}$ formation *in vivo*. Hydroxyl radicals can also be generated from $O_2^{\cdot -}$ and H_2O_2 under *in vitro* incubation conditions when exogenous iron is added. However ADP or ATP-chelated iron react poorly with H_2O_2 to form $OH^{\cdot}$ radical whereas EDTA iron is much more efficient at catalysing this reaction (Vile & Winterbourne, 1987).

4.2.3 Perferryl ion

Reaction of Fe^{3+} with superoxide radical also produces perferryl as an intermediate complex:

$$Fe^{3+} + O_2^{\cdot -} \text{ <---> } [Fe^{3+} - O_2^{\cdot -} \text{ <---> } Fe^{2+} - O_2] \text{ ---> } Fe^{2+} + O_2$$

Thus $OH^{\cdot}$ is not the only reactive species that can be formed in systems containing $O_2^{\cdot -}$ and metal ions.

4.2.4 Hydrogen peroxide

Any biological system generating the superoxide radical will produce hydrogen peroxide by the dismutation reaction, unless all the $O_2^{\cdot -}$ is intercepted by some other molecule. Hydrogen peroxide production has been observed from microsomes *in vitro* (Ernster, et al, 1982). It is, however, extremely difficult to detect *in vivo*.

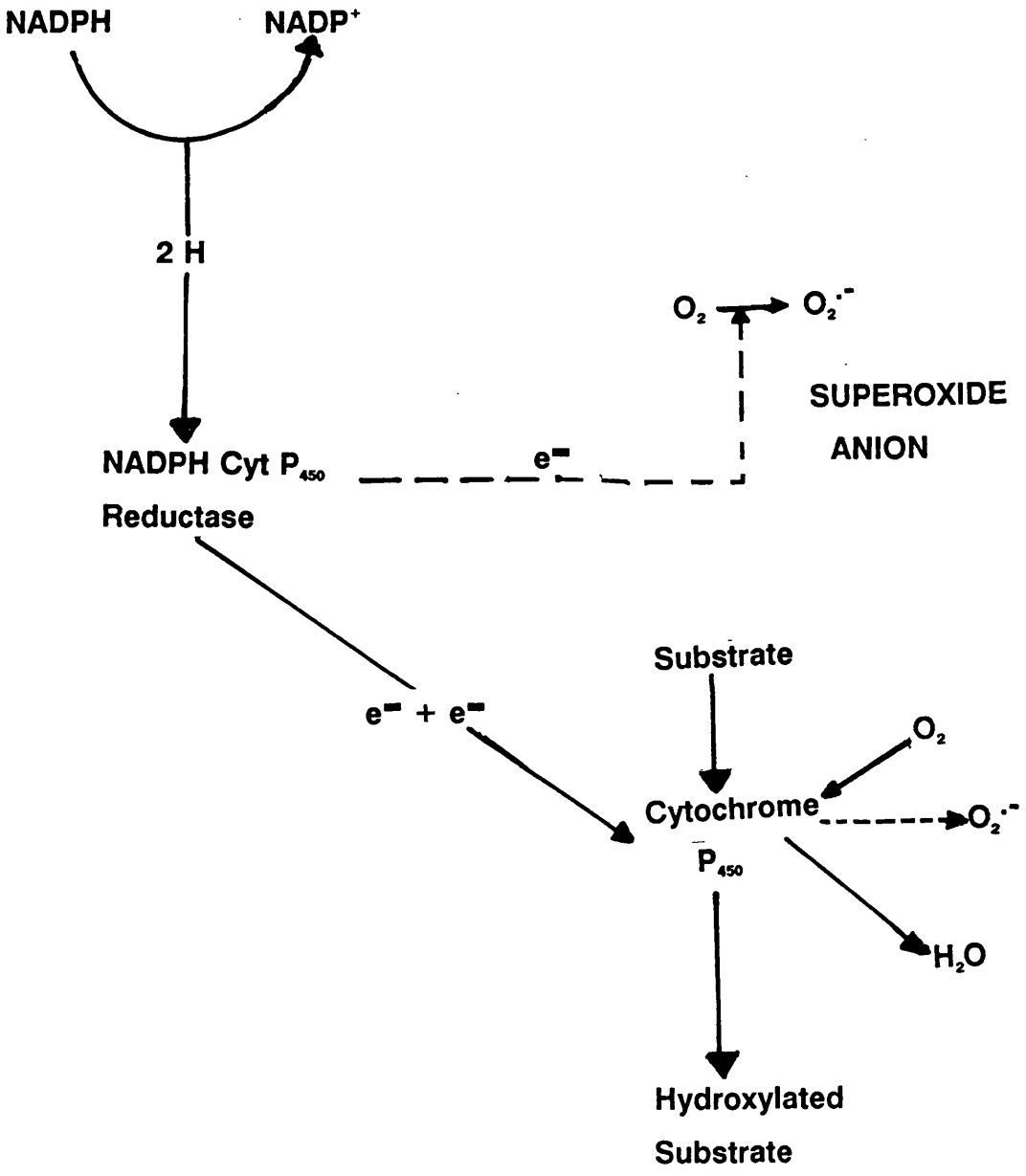
Hydrogen peroxide is a weak oxidising agent which can cross cell membranes rapidly whereas $O_2^{\cdot -}$ usually cannot. Once inside cells, H_2O_2 can inactivate membrane proteins directly by oxidation of essential thiol groups; it is also capable of depleting the antioxidant protective molecule, GSH:

$$H_2O_2 + 2GSH \text{ ---> } GSSG + 2H_2O$$

The reaction is catalysed by glutathione peroxidase and gluthathione transferases. Thus depletion of GSH would make the cell and its organelles more susceptible to oxidative damage. Finally, as shown previously, H_2O_2 can react with Fe^{2+} ions to form the hydroxyl radical and this may be another mechanism for many of its toxic effects. In our incubation system, any H_2O_2 formed, would react with Fe^{2+} and then form hydroxyl radicals. However in the standard assay, this reaction would be inefficient because ADP-chelated iron participates poorly in the iron-catalysed Haber-Weiss reaction (Vile and Winterbourne, 1987). Furthermore, BR was

FIG 4.1

One electron transfer from NADPH cytochrome P_{450} reductase to either cytochrome P_{450} or dioxygen.



normally prepared in dimethylsulphoxide, which is a good scavenger of $OH^{\cdot}$ radicals so that this radical is unlikely to be involved in the degradation of BR.

4.3 ANTIOXIDANT PROPERTY OF BILIRUBIN

All living organisms possess antioxidant defences to prevent the persistance of oxidants as well as to repair damage to tissues and macromolecules. These defences can be enzymatic (SOD, catalase and the glutathione cycle) or non-enzymatic including vitamins E and C and β-carotene (Burton & Ingold, 1984).

Bilirubin is lipophilic and contains an extended system of conjugated double bonds and a reactive hydrogen atom. It could, therefore, theoretically act as a chain breaking antioxidant by a mechanism similar to the well known chain breaking antioxidant vitamin E. Indeed, as a result of studies in which the oxidation of linoleic acid or soyabean phosphatidylcholine was initiated by either 2,2'-azobis (2-4-dimethylvaleronitrile) (AMVN) or 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) respectively, an antioxidant role for unconjugated BR (Stocker et al, 1987), albumin-bound BR (Stocker et al, 1987) and conjugated BR (Stocker & Ames, 1987) have been proposed. According to these authors, bilirubin can scavenge the peroxyl radical ($LOO^{\cdot}$) formed in the LPO process, by donating a hydrogen atom attached to the C-10 bridge of the tetrapyrrole molecule to form a carbon-centered radical ($BR^{\cdot}$) which may undergo further oxidation.

$$LOO^{\cdot} + BR \text{ ----------> } LOOH + BR^{\cdot}$$

The antioxidant property of BR was therefore investigated under the enzymatic incubation conditions described, at various concentrations of BR. Lipid peroxidation activities in hepatic microsomes prepared from Gunn, Wistar and Sprague Dawley rats were compared to see whether the hyperbilirubinaemia of the Gunn rat had an antioxidant effect on hepatic microsomal LPO activity.

4.4 METHODS

4.4.1 Enzymatic and non-enzymatic systems for LPO and BRD activities

Incubations were performed in which hepatic microsomal LPO was initiated either enzymatically or non-enzymatically. Microsomes were also heated for 5 min at 83°C in some experiments (Chapter.3). BRD and LPO activities were determined simultaneously generally after 10 min incubation.

The effect of the antioxidants ascorbic acid and glutathione were investigated. Either 1 mM or 0.2 mM glutathione was added to both the enzymatic and non-enzymatic systems. Non-enzymatic LPO activity was determined with 0.2, 2 and 10 mM ascorbic acid. 10 mM ascorbic acid was also added to the enzymatic system. Activities for LPO and BRD were determined as described in Chapter 3.

4.4.2 The effects of other oxidative species on LPO and BRD.

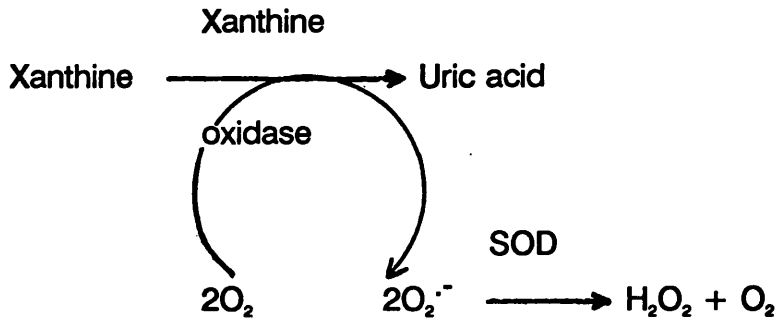
4.4.2.1 SUPEROXIDE ANION RADICAL ($O_2^{\cdot -}$)

a) Initiation studies with superoxide dismutase

Experiments were performed using the enzyme superoxide dismutase from bovine erythrocytes to see whether abolition of superoxide anions influenced LPO and BRD. SOD was added to the standard incubation system to give a final concentration of 33 $\mu g ml^{-1}$. LPO and BRD activities were determined in the presence and absence of SOD.

b) Generation of superoxide anions by xanthine / xanthine oxidase

In a second group of experiments, a model system containing xanthine and xanthine oxidase was used for the generation of $O_2^{\cdot -}$ *in vitro*, to determine the effect of $O_2^{\cdot -}$ on BRD.



The effect of $O_2^{\cdot -}$ on BR degradation without microsomes.

BR (35 μM) prepared in DMSO and taurocholic acid was incubated with xanthine (0.33 mM) and xanthine oxidase (0.04 U ml^{-1}) in 40 mM MOPS buffer pH 7.4. SOD (33 $\mu g ml^{-1}$) was added to some incubations. The reaction was started by the addition of xanthine oxidase. Flasks were incubated in dim light at 37 °C in a shaking water bath. Samples were removed at 0 and 10 min of incubation and assayed for BR.

To examine whether $O_2^{\cdot -}$ initiates LPO and BRD in the presence of hepatic microsomes, Gunn rat liver microsomes (2 mg ml^{-1}) from 4 rats were incubated with BR, xanthine, xanthine oxidase and MOPS buffer as described above, with and without SOD (33 μg ml^{-1}).

4.4.2.2 HYDROXYL RADICAL GENERATION

The mothod employed to generate and assay hydroxyl radical formation was essentially that of Cederbaum and Cohen, (1985). In order to maximize $OH^{\cdot}$ production, EDTA and azide were included in the incubation mixture since EDTA increases $OH^{\cdot}$ formation and azide inhibits catalase and so prevents the conversion of hydrogen peroxide to water and oxygen. DMSO was added as part of the BR preparation and reacted with $OH^{\cdot}$ radicals to give methyl radicals, which produce formaldehyde that can be detected colorimetrically:

(i) $(CH_3)_2$-SO + $OH^{\cdot}$ ⟶ CH_3SOOH + $CH_3^{\cdot}$

methyl radical

(ii) $CH_3^{\cdot}$ ⟶ CH_4 methane

CH_3-CH_3

ethane

CH_2O

formaldehyde

An hydroxyl radical generating system consisting of hepatic microsomes (1.5 - 2 mg ml^{-1} protein), 0.1 mM EDTA, 18 μM $FeSO_4$, 1 mM azide, together with an NADPH generating system (described in Chapter 3) and BR (35 μM) dissolved in DMSO (100 mM) and MOPS (40 mM) buffer pH 7.4 containing sodium taurocholate (1.67 mM), was used to observe the effect of hydroxyl radicals on NADPH initiated LPO and BRD. Incubations were carried out at 37°C and samples were taken at various incubation times for determinations of LPO and BRD and for hydroxyl radical formation by the Nash reaction (Nash, 1953).

The incubation mixture was deproteinised by mixing with an equal volume of 0.9 % trichloracetic acid, and centrifuged at 2,500 rpm for 10 min. The supernatant was used for the formaldehyde determination (Nash, 1953) as follows. Equal volumes of deproteinised incubation mixture and Nash reagent (2 M ammonium acetate, 0.02 M acetylacetone and 0.05 M glacial acetic acid) were heated at 60 °C for 10 min. Tubes were cooled and absorbance read at 415 nm in a spectrophotometer.

4.4.2.3 EFFECT OF HYDROGEN PEROXIDE

Addition of H_2O_2

To determine the effect of H_2O_2 on BRD, H_2O_2 (30 %) was initially added to the complete system plus and minus the cofactors. A violent reaction occured when microsomes were added and BR was rapidly bleached.

In subsequent experiments, H_2O_2 was added to microsomes, BR and buffer firstly

in serial dilutions to give final concentrations of 113.6, 11.36, 1.136 and 0.114 mM. Finally 2.3 M and 1.14 mM H_2O_2 were added to standard incubations containing microsomes, BR and buffer with and without ADP / $FeSO_4$. NADPH generating system was only included in the incubation where 2.3 M H_2O_2 was added. Reactions were started by the addition of H_2O_2.

Addition of Catalase

The enzyme catalase catalyses the reaction:

$$2H_2O_2 \text{ ------> } 2H_2O + O_2$$

The addition of catalase to the normal incubation system would therefore prevent any hydrogen peroxide accumulation. Incubations were therefore performed as described previously (Chapter.3), in the presence or absence of thymol-free catalase (2,500 units ml^{-1} final conc.). LPO and BRD activities were determined.

4.4.3 Investigation of the lipid antioxidant property of bilirubin

Three sets of experiments were performed:

1 In order to investigate whether BR was acting as an antioxidant in the enzymatically initiated microsomal LPO system, increasing concentrations of BR were added (1-65 μM) to the standard incubation system and LPO was monitered.

2 Standard incubations were performed at 2 % oxygen (method is described in Chapter 3) in the presence and absence of BR (36 μM) and LPO activity measured.

3 A comparision of enzymatically initiated microsomal LPO activities in the absence of exogenous bilirubin, between the normal Wistar and Sprague Dawley strains of rats and the jaundiced Gunn rats was made.

4.5 RESULTS AND DISCUSSION

4.5.1 Enzymatic and non-enzymatic LPO and BRD

A time course for LPO and BRD initiated enzymatically, (Fig 4.2), shows that as BR is degraded, LPO progresses in parallel. Parallelism between the two activities has also been demonstrated for the non-enzymatic system (data not shown). Heat treatment of microsomes abolished both LPO and BRD in the enzymatic system, whereas it had no significant effect in the non-enzymatic system (Fig 4.3). These data support the fact that the enzymatically initiated LPO mechanism is indeed an enzymatic process since the enzymes become denatured when heated resulting in the abolition of LPO and BRD activities. In contrast, heat-treatment failed to abolish these activities in the iron-ascorbate initiated system confirming that the mechanism involved in this case is non-enzymatic.

In order to assess the relation between LPO and BRD a plot of these activities in a series of experiments is presented in Figs 4.4 and 4.5.
A significant correlation between LPO and BRD activities was observed for the enzymatic system ($r = 0.78$, $p < 0.001$, $n = 23$) and non-enzymatic system ($r = 0.71$, $p < 0.02$, $n = 11$). This suggests that BR is oxidised as LPO proceeds.

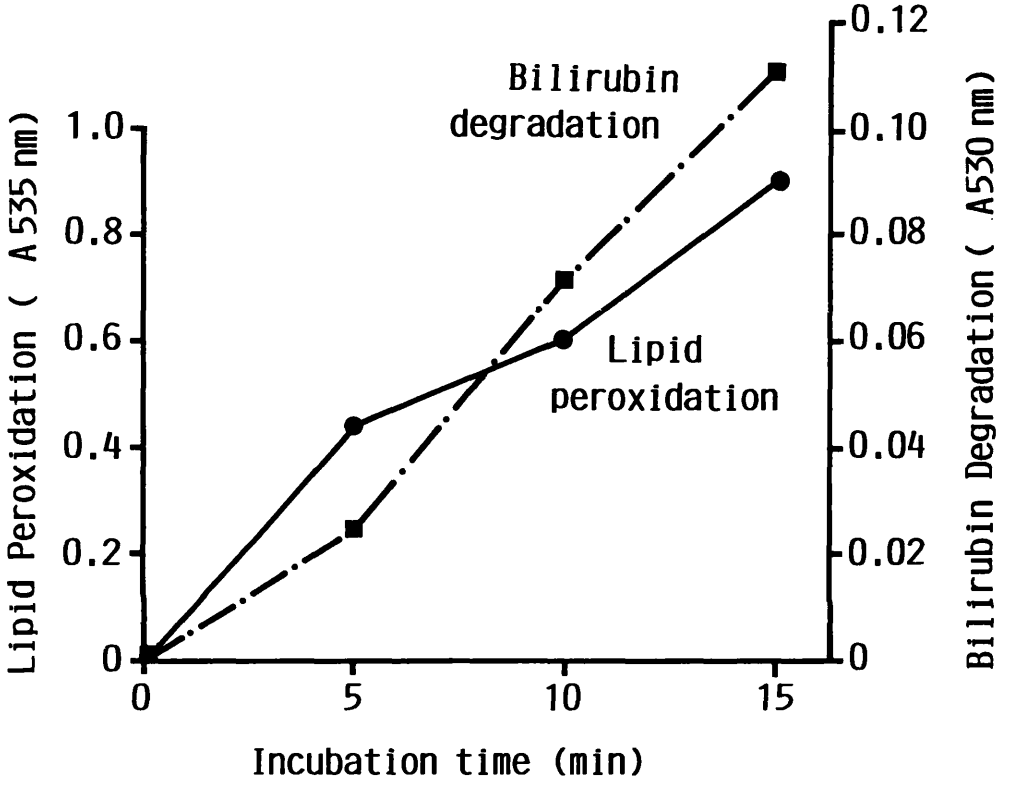
Effect of ascorbic acid on LPO

Fig 4.6 shows that in the absence of an NADPH-generating system, LPO is initiated non-enzymatically by 0.2 mM ascorbic acid; with 2 mM ascorbate, considerable inhibition of LPO (and BRD not shown) occurs and with 10 mM ascorbate LPO and BRD activities were completely abolished. 10 mM ascorbic acid was also found to completely inhibit enzymatic LPO and BRD activities (data not shown).

These findings confirm previous observations (Wills, 1969, Heikkila & Manzino, 1987) that ascorbic acid initiates LPO at low concentrations (0.06 to 0.6 mM) while at high concentrations (6 mM), it has an inhibitory effect (Heikkila & Manzino, 1987). Ascorbic acid is a relatively good reducing agent and it is often used to

FIG 4.2

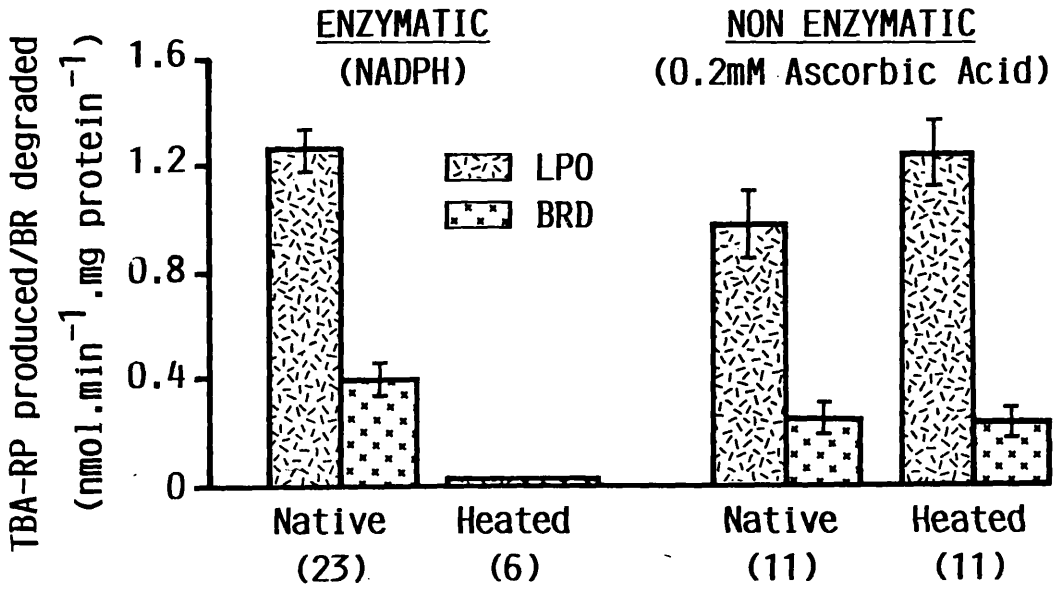
ENZYMATIC LIPID PEROXIDATION IN HEPATIC MICROSOMES : EFFECT ON EXOGENOUS BILIRUBIN



Microsomes were incubated as described in Chapter 3 with 35 μM BR and 0.5 ml samples of the incubate were removed at 0, 5, 10 and 15 min for the assay of lipid peroxidation (A 535 nm, ●-●) and bilirubin degradation (A 530 nm, ■-■). LPO was determined as the increase in formation of the thiobarbituric acid reactive product, while BRD was assessed as the decrease in absorbance of the diazotised BR.

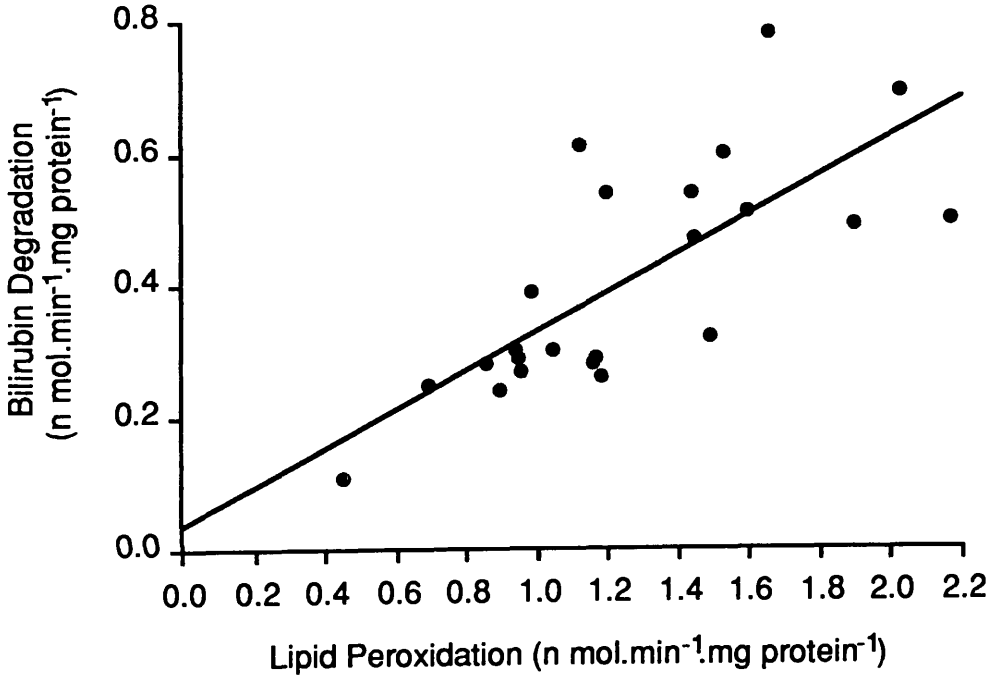
FIG 4.3

HEPATIC MICROSOMAL INDUCTION OF LIPID PEROXIDATION AND BILIRUBIN DEGRADATION (Mean ± SEM)



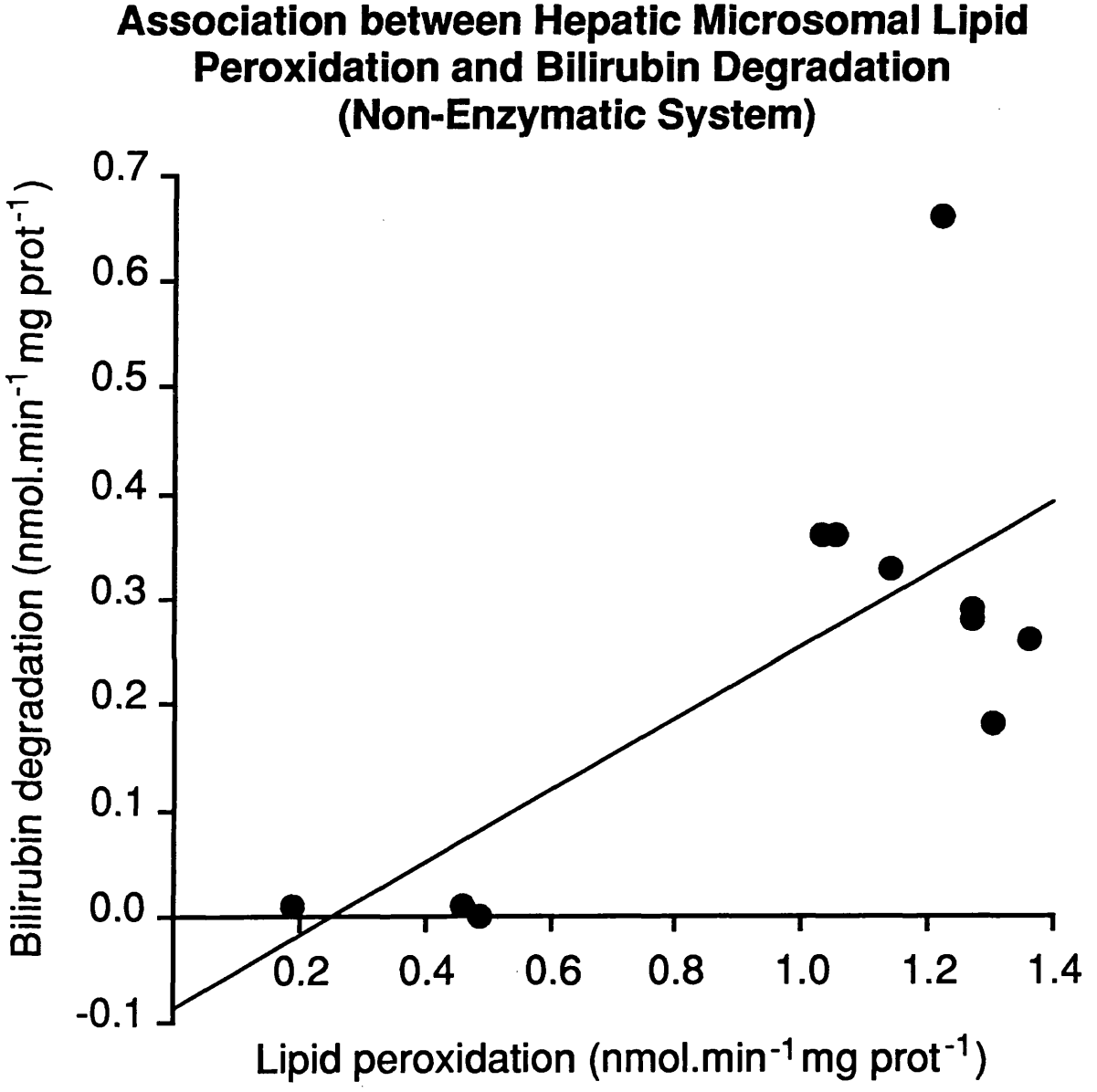
Gunn rat liver microsomes were resuspended and either kept on ice (native) or subjected to heat treatment at 83-85 °C for 5 min before incubation for 10 min with either NADPH or ascorbic acid as described previously (Chapter 3). LPO and BRD activities were expressed as nmol TBA-RP formed (LPO) or nmol BR degraded (BRD), per min per mg protein.

Association Between
Hepatic Microsomal Lipid Peroxidation
FIG 4.4 and Bilirubin Degradation (Enzymatic System)



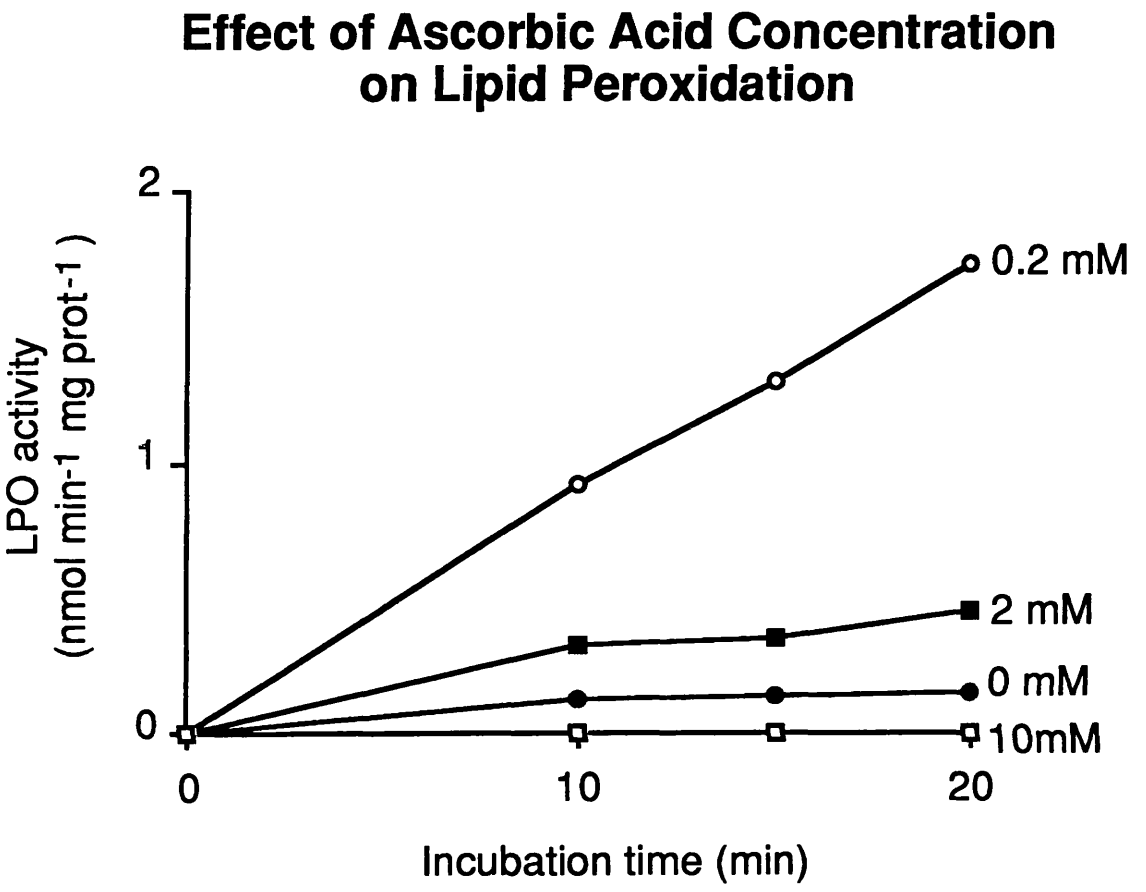
Scatter diagram of LPO and BRD initiated enzymatically showing a linear regression line, y = 0.29x + 0.03, (n = 23, r = 0.78, p < 0.001). The activities were calculated after 10 min.

Fig 4.5



Scatter diagram of LPO and BRD initiated non-enzymatically showing linear regression line, y = 0.34x - 0.09, (n = 11, r = 0.71, p < 0.02). The activities were calculated after 10 min incubation.

Fig 4.6



Hepatic microsomes were incubated at 37 °C in 40 mM MOPS buffer, pH 7.4 with 18 μM $FeSO_4$ and 1.6 mM ADP and ascorbic acid (0-10 mM) to initiate LPO and BRD non-enzymatically. Samples of the incubate were removed at 0, 10, 15 and 20 min and TBA-RP assays performed in order to determine LPO activity.

TABLE 4.1 EFFECT OF GLUTATHIONE (1mM) ON ENZMATICALLY AND NON-ENZYMATICALLY INITIATED LPO AND BRD ACTIVITIES IN NATIVE AND HEAT-TREATED MICRCOSOMES

	n	Mean activity ± SEM (nmol min^{-1} mg $prot^{-1}$)	
NON-ENZYMATIC SYSTEM		LPO	BRD
Native microsomes minus GSH	11	0.98 ± 0.12	0.25 ± 0.06
Native microsomes plus GSH	6	0.49 ± 0.12*	0.04 ± 0.03
Heat-treated microsomes minus GSH	11	1.24 ± 0.13	0.26 ± 0.13
Heat-treated microsomes plus GSH	6	1.01 ± 0.11	0.19 ± 0.07
ENZYMATIC SYSTEM			
Native microsomes minus GSH	23	1.26 ± 0.09	0.40 ± 0.04
Native microsomes plus GSH	4	0.66 ± 0.04*	0.19 ± 0.02

* A significant difference in LPO and BRD activities was observed in native microsomes in both the non-enzymatic and enzymatic systems ($p<0.01$). There was no significant difference in activities for heat-treated microsomes.

prevent unwanted oxidation processes. However, in view of the fact that at low concentrations in the presence of iron, it can stimulate oxidative decomposition, care must be taken with its indiscriminate use as a reducing agent.

Inhibition of LPO and BRD by glutathione

In preliminary experiments, glutathione was found to have an inhibitory effect at 0.2 mM and 1 mM final concentrations while at 10 mM it abolished LPO and BRD completely (data not shown). In both enzymatic and non-enzymatic systems, 1mM GSH produced partial inhibition of LPO at 10 min incubation (Table 4.1). Results showed that on heat treatment of microsomes, the inhibitory effect of GSH was almost completely abolished, suggesting that GSH must be inhibiting LPO in native microsomes via an enzymatic mechanism, which is destroyed on heat treatment. The physiological hepatic GSH concentration for rat is 7-8 mM (Halliwell and Gutteridge, 1989) so these results suggest an antioxidant role for this molecule *in vivo*. It is of interest to note that an enzyme catalysed synergism between glutathione and vitamin E has recently been demonstrated (Leedle & Aust, 1990). Glutathione was shown to regenerate vitamin E from its radical thus inhibiting LPO.

These *in vitro* studies support the hypothesis that the degradation of BR is an oxidative process dependent on LPO.

4.5.2 Superoxide anion radicals

Experiment 1

Addition of SOD to the normal enzymatic system did not produce a significant difference in LPO and BRD activities (Table 4.2). This finding suggests that superoxide anions that are accessible to exogenous SOD are not involved in NADPH-dependent LPO and confirms previous work by Pederson and Aust (1975).

Experiment 2

In the absence of microsomes, the superoxide anions generated by the xanthine

/ xanthine oxidase system degraded BR at rates comparable to those obtained with the microsomal systems (Table 4.3). SOD inhibited superoxide mediated BRD by 85%. These findings confirm those of Broderson and Bartels (1969) and Kaul et al (1979) who studied the degradation by superoxide of bilirubin in simple buffered solutions.

However when the same xanthine / xanthine oxidase system was added to hepatic microsomes from Gunn rats, both LPO and BRD activities were zero (Table 4.3). Superoxide anions had failed to initiate LPO in microsomes and also failed to degrade BR directly. Under identical conditions, microsomes caused only a modest decrease (10-20 %) in superoxide formation by the xanthine / xanthine oxidase system (R.Stanley & T.Hallinan, unpublished data).

Since SOD failed to inhibit LPO initiated by the NADPH enzymatic mechanism and in addition the xanthine / xanthine oxidase system, which was capable of degrading bilirubin, failed to do so in the presence of microsomes, it seems unlikely that superoxide anions play a role in the microsomal oxidation of BR in the system studied, or influence LPO activity.

4.5.3 Hydroxyl radical

Negligible amounts of hydroxyl radicals were detected under normal assay conditions (Table 4.4). When conditions to optimize hydroxyl radical generation were used, LPO and BRD activities were trivial. It can therefore be concluded that hydroxyl radicals are not involved in either initiating LPO or degrading BR in the *in vitro* assay by a mechanism independent of LPO.

4.5.4 Hydrogen peroxide

There was no difference in LPO and BRD activities in the presence of catalase, (Table 4.5) suggesting that hydrogen peroxide, which is accessible to exogenous catalase, is not responsible for LPO or BRD under the conditions used. If it had an effect on BRD this would have been independent of LPO.

TABLE 4.2 EFFECT OF SOD ON LPO AND BRD ACTIVITIES IN THE ENZYMATIC SYSTEM

Assay conditions	n	Mean activities ± SEM (nmol min^{-1} mg $prot^{-1}$)	
		LPO	BRD
Standard	5	1.22 ± 0.08	0.28 ± 0.02
Standard plus SOD (33μg ml^{-1})	7	1.30 ± 0.05	0.26 ± 0.03

TABLE 4.3 EFFECT OF SUPEROXIDE ANIONS ON BR IN THE PRESENCE AND ABSENCE OF MICROSOMES

	n	Mean activity ± SEM (nmol min^{-1}) LPO	BRD
BR + xanthine/ xanthine oxidase	2	-	0.45 (0.48, 0.42)
BR + xanthine/ xanthine oxidase + SOD	2	-	0.07 (0.04, 0.09)
BR + xanthine/ xanthine oxidase + microsomes	4	0	0
BR + xanthine/ xanthine oxidase + microsomes + SOD	4	0	0

BR (35 μM), Xanthine (0.33 mM), Xanthine Oxidase (0.04 U ml^{-1}), SOD (33 μg ml^{-1}), microsomal prot. conc. 2 mg ml^{-1}, 40mM MOPS.

Direct addition of hydrogen peroxide to microsomes, BR and buffer showed that at low concs (0.1 - 11.4 mM), BRD activity was negligible (data not shown). In 4 experiments with the standard assay conditions minus NADPH generating system, a concentration of 1.14 mM H_2O_2 produced insignificant LPO and BRD activities (Table 4.5). At higher H_2O_2 concentrations (2.3 M), which interfered with the LPO assay, marked BRD activity was attained, suggesting non-enzymatic oxidation of BR. Since the physiological concentration of H_2O_2 in liver is about 1 μM (Chance et al, 1979), it is unlikely that H_2O_2 plays an oxidative role in BRD *in vivo*.

4.5.5 Antioxidant property of bilirubin

The present investigations show that a range of bilirubin concentrations, did not have an antioxidant effect on LPO in Gunn rat liver microsomes (Fig 4.7 a & b). In contrast, Wolfram et al, 1986, using rat liver microsomes, have reported that both bilirubin and biliverdin inhibit LPO at concentrations of 5 or 10 μM. These authors suggested that the bile pigments decreased the initial rate of LPO by scavenging superoxide radicals. However since superoxide radicals do not appear to be responsible for the initiation of LPO in the rat liver microsomal system (Pederson & Aust, 1975 and Table 4.3), bilirubin would not be expected to have an inhibitory effect by this mechanism. Why Wolfram's findings differ from our own is still a matter for conjecture.

Bilirubin has been reported to scavenge lipid peroxyl radicals generated chemically, thus preventing LPO (Stocker et al, 1987). This antioxidant activity increased as the oxygen concentration was decreased from that in air (20%) to a physiologically relevant tissue concentration (2%). However, in the present study, even when experiments were performed in 2 % oxygen, there was no evidence of significant inhibition of LPO (Fig 4.8). Moreover, microsomal LPO activity for Gunn rats in the absence of exogenous bilirubin, was not less than that in Wistar and Sprague Dawley rats (Fig 4.9) in spite of the fact that the concentration of bilirubin in the Gunn microsomal membranes might be expected to be higher. If bilirubin is oxidized in the incubation system described, then it must be reducing the species that is oxidising it and thus acting as an antioxidant. The microsomal system was designed to observe degradation of BR, the resultant measured LPO

TABLE 4.4 EFFECT OF HYDROXYL RADICALS ON ENZYMATIC MICROSOMAL LPO AND BRD ACTIVITIES

	$OH^{\cdot}$ formation (nmol min^{-1} mg $prot^{-1}$)	Mean activities ± SEM (nmol min^{-1} mg $prot^{-1}$)	
		LPO	BRD
Standard assay conditions	0.06 ± 0.03 (n=3)	1.21 ± 0.08 (n=5)	0.34 ± 0.04 (n=3)
$OH^{\cdot}$ generating system	0.89 ± 0.03 (n=6)	0.00 (n=6)	0.01 ± 0.01 (n=5)

Standard enzymatic conditions are described in chapter 3. The $OH^{\cdot}$ generating system consisted of microsomes (1.5-2 mg ml^{-1} prot), 0.1 mM EDTA, 18μM $FeSO_4$, 1 mM azide, NADPH generating system (as for the standard assay) and DMSO (100 mM). BR (35 μM) in MOPS (40 mM) buffer pH 7.4 containing sodium taurocholate (1.67 mM) was added to observe the effect of $OH^{\cdot}$ on BRD (section 4.4.2)

TABLE 4.5 EFFECT OF ADDED H_2O_2 OR CATALASE ON ENZYMATIC LPO AND BRD ACTIVITIES

Assay Conditions	n	Mean activities ± SEM (nmol min^{-1} mg $prot^{-1}$) LPO	BRD
Standard	6	1.38 ± 0.14	0.31 ± 0.03
Standard plus* 1.14 mM H_2O_2	4	0.02 ± 0.01	0.08 ± 0.05
Standard plus** 2.3 M H_2O_2	1	-	1.32
Standard plus catalase 2,500 U ml^{-1}	5	1.38 ± 0.11	0.36 + 0.02

* H_2O_2 (1.14 mM) was added to the standard microsomal enzymatic system without NADPH.

** LPO in the presence of 2.3 M H_2O_2 could not be assayed; the high conc of H_2O_2 interfered with the LPO assay. In the absence of $FeSO_4$ and ADP similar results were obtained.

Catalase (2,500 U ml^{-1}) was added to the standard incubation system.

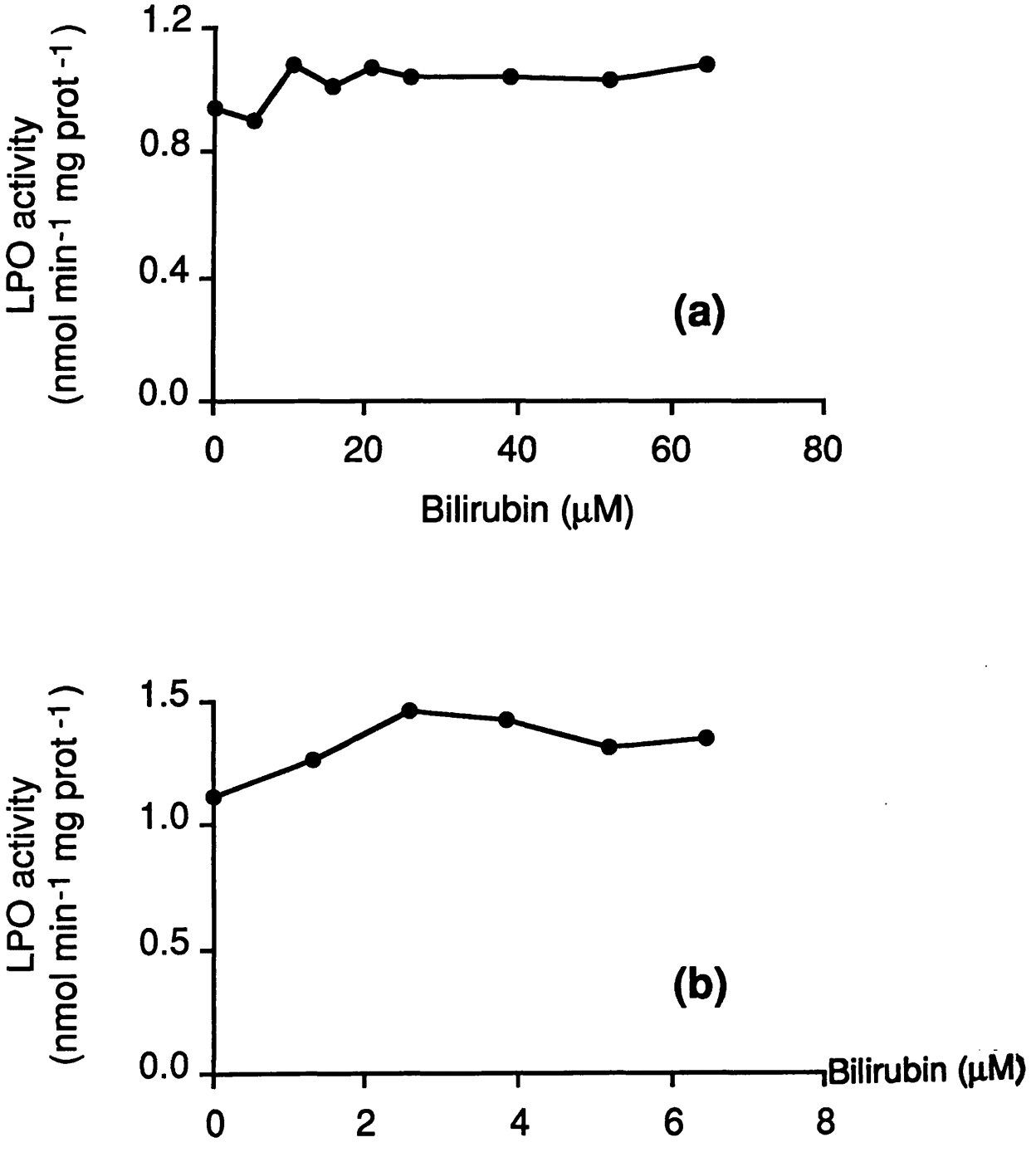
activity was found to be at least four times greater than the BRD activity. However, the MDA produced when assayed as TBA-reactive products, represents about 53% of the carbonyls actually formed (Halliwell & Gutteridge, 1989). The LPO oxidant effect on BR is therefore, dominant over the antioxidant effect of BR on LPO. It is therefore perhaps not surprising that one fails to observe an antioxidant effect by BR in the microsomal system studied which differs from the Stocker (1987) model, not only in the source of lipid, the initiator of LPO and the form of BR added, but also in the products of the reaction. Biliverdin was formed with AAPH induced oxidation of BR-albumin,(Stocker et al, 1987) whereas the microsomal LPO system gave rise to colourless diazo negative products as a result of BR oxidation. The physiological role for BR as an antioxidant remains to be established.

4.6 CONCLUSION

A close association has been demonstrated between lipid peroxidation and bilirubin degradation in the hepatic microsomal systems studied. Other investigators have shown that bilirubin can be degraded chemically by hydrogen peroxide (Brodersen & Bartels, 1969, De Matteis et al, 1989) and superoxide anions (Kaul et al, 1979). However, the present experiments have demonstrated that NADPH-dependent LPO of rat liver microsomes and the consequent BRD, depends on a reaction mechanism that does not require hydroxyl radicals, hydrogen peroxide or superoxide anions. In the NADPH-dependent Gunn rat microsomal system, the addition of catalase did not inhibit LPO, thus providing evidence against the involvement of either hydroxyl radicals or hydrogen peroxide in the initiation mechanism. Furthermore, an hydroxyl radical generating system failed to initiate LPO though this could perhaps have been due to inadequate hydroxyl radical production and the presence of EDTA in the incubation medium. Both hydroxyl radicals and superoxide anions have been shown to initiate LPO in pure lipid systems (Galeotti & Borrello, 1988). However, data from the present studies argues against superoxide anions being the reactive oxidative species responsible for the initiation of LPO in microsomes (Tables 4.4 & 4.5) and this confirms previous findings with respect to the initiation of microsomal LPO (Pederson & Aust, 1975; Svingen et al, 1979).

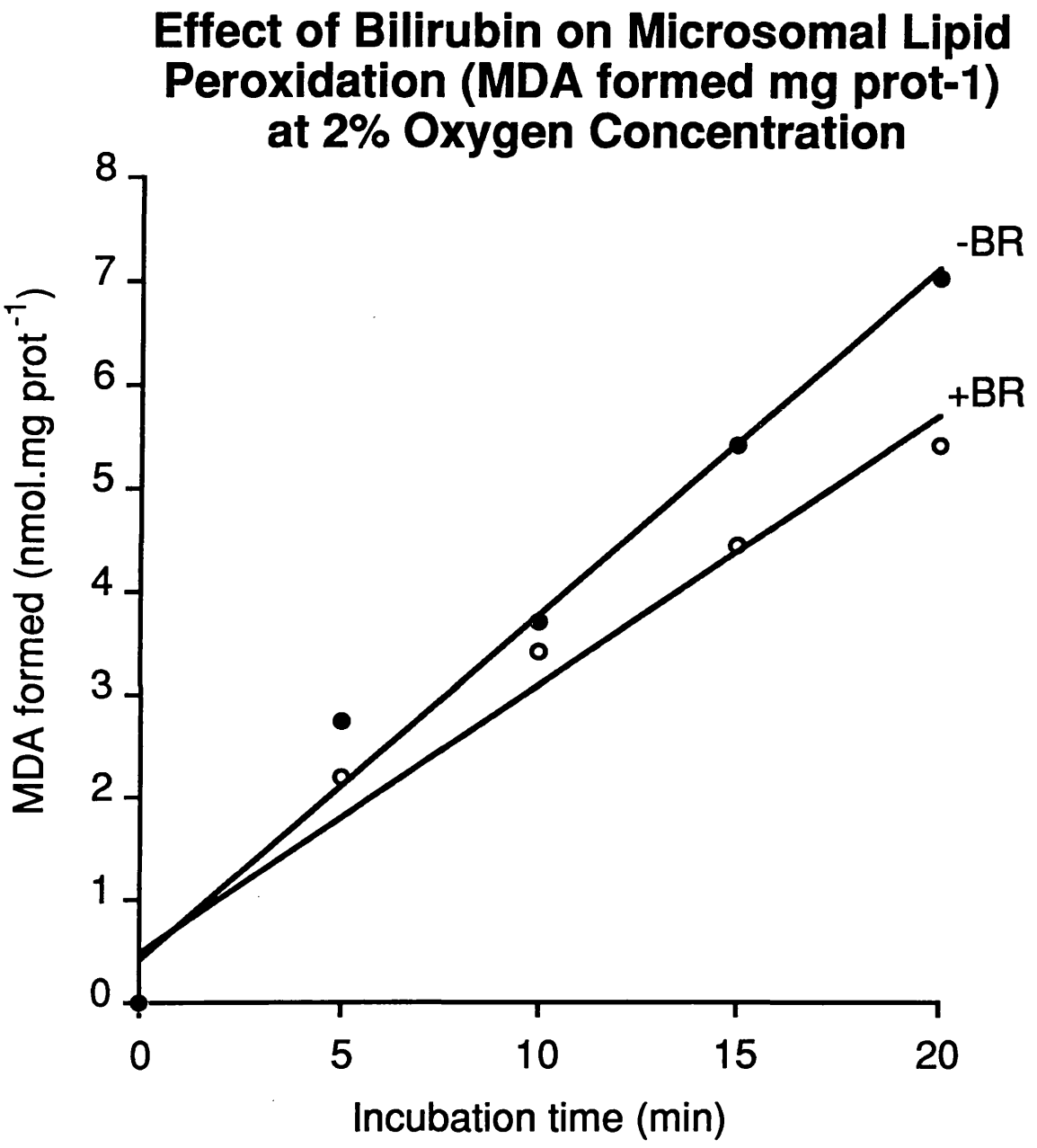
Fig 4.7

Effect of Bilirubin on Enzymatically Initiated Lipid Peroxidation



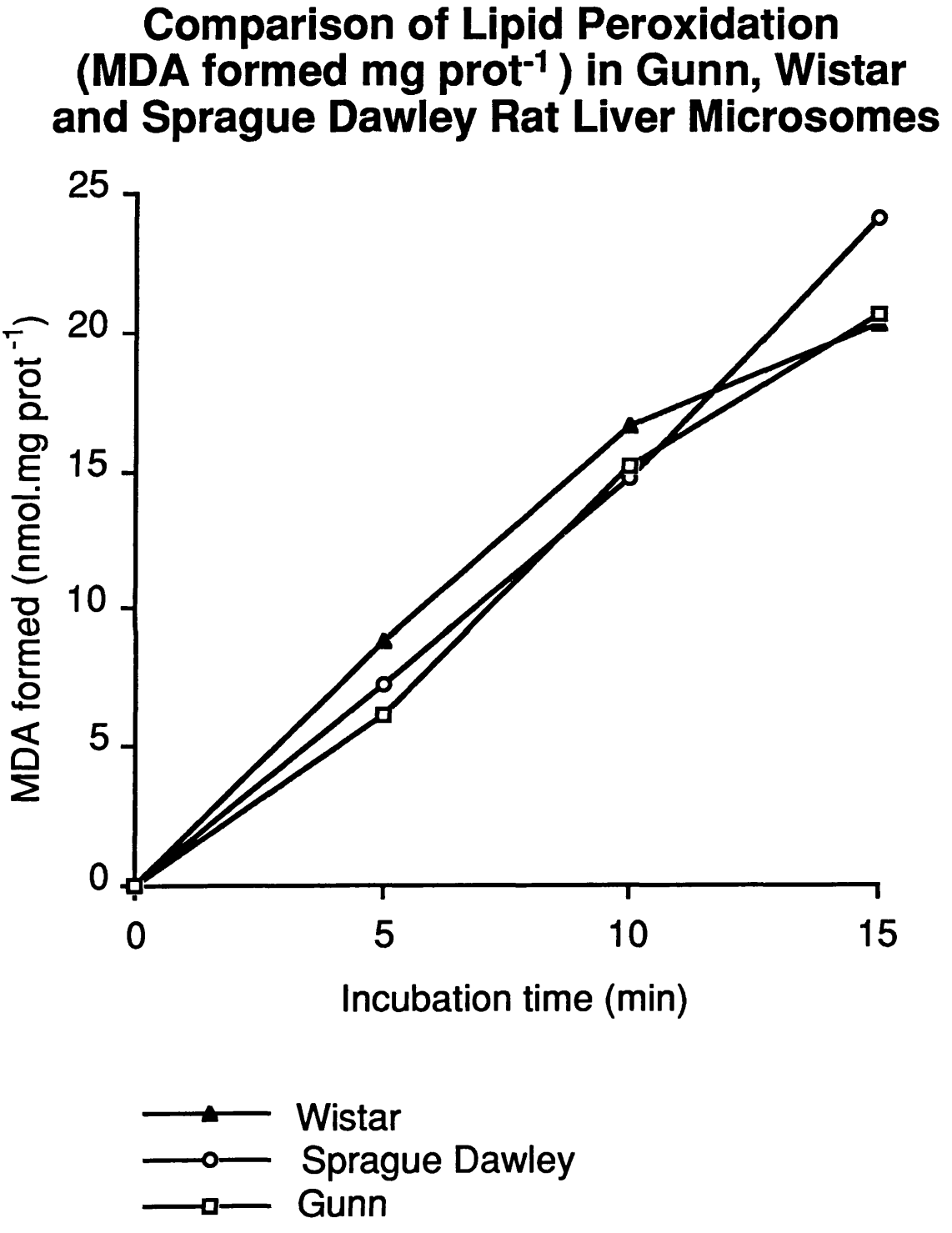
Bilirubin (0-70 μM (a)) and (0-8 μM (b)), was added to the enzymatic LPO system and samples were removed at 0 and 10 min for LPO activity determinations.

Fig 4.8



Hepatic microsomal LPO activity was assayed in 2 % oxygen (98 % N_2) at various incubation times (0-20 min) in the presence or absence of bilirubin (35 μM) in the enzymatic system. The activities observed in the presence and absence of BR were not significantly different.

Fig 4.9



Hepatic microsomal LPO activity was compared in Gunn, Wistar and Sprague Dawley rats under standard enzymatic conditions after incubations of 0, 5, 10 and 15 min in the absence of exogenous BR.

The mechanism for LPO in both the enzymatic and non-enzymatic systems, showed an absolute requirement for iron and oxygen (Chapter 3). It is possible, therefore that LPO is initiated by some form of iron-oxygen complex generated during the oxidation of iron (II) by oxygen. The involvement of perferryl complexes was first suggested by Hochstein and Ernster (1964). Complementing this theory, Aust et al, (1985) have proposed that the initiation mechanism requires an iron II / iron III / oxygen complex or some critical ratio of iron II to iron III. However, attempts to characterize this complex have been unsuccessful. In addition, transition metal complexes have been shown to stimulate LPO by accelerating the decomposition of lipid hydroperoxides to peroxyl ($ROO^{\cdot}$) and alkoxyl radicals ($RO^{\cdot}$).

In summary, it is possible that an iron-oxygen complex is one of the oxidative species responsible for the initiation of LPO *in vivo*. However, until an accurate method for measuring LPO *in vivo* is established and the conditions controlling its activity *in vivo* determined, the exact mechanism involved in its initiation will continue to be a subject for debate.

CHAPTER 5

IN VIVO STUDIES

5.1 DIETARY MODULATION OF PLASMA BILIRUBIN CONCENTRATION.

5.1.1 INTRODUCTION

It has been shown that dietary lipid lowers the plasma bilirubin in Gunn rats, while a lipid free diet elevates it (Housset et al, 1967, Gollan et al 1975, Gollan et al 1979). These findings have not been satisfactorily explained. Since the *in vitro* studies discussed in Chapter 4 showed a good correlation between hepatic microsomal lipid peroxidation and bilirubin degradation, it was the aim of experiments in this chapter to determine whether dietary modulation of plasma bilirubin could be accounted for by changes in microsomal lipid peroxidation activity.

It is now well established that the hepatic microsomal cytochrome P_{450} system has two major roles: (i) a physiological role in the biosynthesis and metabolism of cholesterol, bile acids, steroid hormones, prostaglandins, vitamin D etc and (ii) a detoxification role in which it constitutes a protective system against a variety of drugs, pesticides, carcinogens and other xenobiotics, by increasing their polarity and providing functional groups for subsequent conjugation and excretion, as well as oxidative degradation.

The detoxification system can give rise to reactive intermediates which interact with vital cellular macromolecules. Many carcinogens such as polycyclic aromatic hydrocarbons, aromatic amines and aflatoxin, as well as drugs such as

paracetamol, express their toxicity only after oxidative metabolism (Ioannides et al 1984).

The existence of the multiple forms of cytochrome P_{450} has been extensively demonstrated (Lau & Strobel 1982, Guengerich et al 1982, Astrom & DePierre 1985) and at least eleven different forms have been purified from rat liver microsomes and shown to differ in substrate specificity, absorbance maximum of the carbon monoxide complex of the reduced cytochrome, molecular weight, amino acid composition, immunochemical properties, and peptide maps. Therefore, individual forms of cytochrome P_{450} can be induced by different compounds and a single compound can lower the level of one form and induce one or more other forms (Guengerich et al 1982).

The induction of cytochrome P_{448} activity has been associated with the mutagenic and carcinogenic activity of the inducing agent (Ioannides et al 1984) and has therefore been suggested to be a useful index of the potential carcinogenicity or toxicity of chemicals whose activation is mediated by the mixed-function oxidase system (Iwasaki et al 1986).

Specific inducers of cytochrome P_{448} such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methyl cholanthrene, and β-napthoflavone have been shown to produce a marked decrease in plasma BR concentration in Gunn rats (Kapitulnik & Ostrow, 1977, Cardenas-Vasquez et al 1986, De Matteis et al 1989,). A marked constitutive increase in the microsomal content of cytochrome P_{448} (cytochrome P_{450c}) has been reported in Gunn rats (Kaptulnik et al, 1987). These findings led to the suggestion that this specific cytochrome P_{448} isoenzyme may be involved in the alternate pathway for BR metabolism in the Gunn rat. It was therefore of interest to measure cytochrome P_{448} activity in hepatic microsomes from Gunn rats maintained on the different lipid diets (see below).

For many years, it was generally assumed that although the fatty acid composition

of adipose tissue and other stored triglycerides can fluctuate according to changes in the fatty acid composition of the dietary lipid, that of the phospholipids of cell membranes does not change to any significant extent. However, it has now been demonstrated in rats that the polyunsaturated fatty acid composition of the diet regulates the fatty acid composition of the liver endoplasmic reticulum and that this is an important factor controlling the rate and extent of lipid peroxidation *in vitro* (Hammer & Wills 1978). Support for the dietary regulation of membrane phospholipids of intracellular organelles of various tissues has also been provided by Daum (1985) and Rogel & Watkins (1987), using rat mitochondria and chick liver microsomes respectively.

In order to determine whether the peroxidizability of the microsomal membrane was related to the lipid in the diet in our experiments, the total double bond index (DBI) for phospholipid fatty acids (PLFAs) was determined by isolation of PLFAs followed by hydrolysis of the fatty acids and separation on GLC. The percentage composition of PLFAs of microsomes from rats on the different diets was used to calculate their DBIs.

The importance of vitamin E (α-tocopherol) in preventing LPO will be discussed later. It has been well documented (Niki, 1987a) and we have confirmed in our studies that vitamin E inhibits lipid peroxidation. It is a lipid soluble antioxidant and its effect would therefore be expected to be related to the lipid content of the membrane. Corn oil used in these studies was estimated to contain 60.9 μg vitamin E per gram, therefore the vitamin E intake by rats on the different diets varied (Table 5.6). Vitamin E concentration has also been demonstrated to depend on other antioxidant systems such as ascorbic acid and glutathione (Graham et al 1989). It was therefore of interest to determine the vitamin E concentration of hepatic microsomes to see whether it altered in relation to the lipid peroxidation activity measured *in vitro*.

5.1.2 METHODS

5.1.2.1 DIETS

Gunn rats were maintained on either standard laboratory diet (SDS / BP; Table 5.1) or a synthetic diet prepared as described by The Department of Nutrition, Queen Elizabeth College, the composition of which is shown in Table 5.2. Solkafloc, a wood cellulose product used as a source of dietary fibre in the synthetic diet, was obtained from Special Diets Services Ltd., Witham Essex.

5.1.2.2 DIETARY REGIMEN.

Male Gunn rats (280-380g) were fed for 1 week on the standard diet, in powdered form so that they would become accustomed to eating their food in this form. They were then fed for 2 weeks ad libitum on diets containing either 0 %, 8 %, or 25 % w/w corn oil, obtained from Budgens (Table 5.3). Blood samples were obtained under halothane anaesthesia by cardiac puncture at the beginning and end of the test period and plasma bilirubin concentrations were determined by a standard diazo method with caffeine as the accelerator (Michaelsson et al 1965). The initial BR concentrations ranged from 129-310 μM (mean 179 $\pm$ 8, n = 24). After 14 days, the rats were sacrificed and liver microsomes were prepared by the rapid calcium aggregration method described in Chapter 3 and stored at -70 °C.
Microsomes from rats maintained on the three diets were used in the following studies:

(i) Measurement of LPO and BRD activities in enzymatically and non-enzymatically initiated *in vitro* assays as described in the previous chapter. Non-enzymatically initiated LPO and BRD activities were also determined in heat-treated microsomes (83 °C, 5 min).
(ii) Assay of cytochrome P_{448} activity.
(iii) Determination of phospholipid fatty acid (PLFA)

TABLE 5.1 STANDARD DIET COMPOSITION

	% (w/w)
Crude oil	2.6
Crude protein	14.7
Starch	45.8
Sugar	6.5
Ash	5.9
Total Dietary Fibre	14.5
Moisture	10.0

Standard diet (SDS/BP) purchased from Special Diets Services, Witham, Essex, contained 68.3 mg α-tocopherol kg^{-1} diet.

TABLE 5.2 COMPOSITION OF THE SYNTHETIC DIET USED IN DIETARY STUDIES

		Weight g/kg diet	
	Corn starch	Varied	
	Corn oil	Varied	
	Casein	200	
*	Mineral mixture	40	
**	Vitamin mixture	20	(α-tocopherol 76 mg kg^{-1} diet)
	Solkafloc	50	

* Mineral mixture (QEC)

	(g)
$CaHPO_4$	2,600
$CaCO_3$	1,640
KCl	1,640
Na_2HPO_4	1,480
$MgSO_4$ H_2O	560
+ Trace minerals	80

** Vitamin mixture (QEC)

	(g)
Solkafloc	1,760
Choline bitartrate	180
++ Vitamin concentrate	60.2

\+ Trace minerals

	(g)
$MnSO_4$, H_2O	180
Fe citrate	174
$CuSO_4$	15
$ZnCO_3$	30
KIO_3	1

++ Vitamin concentrate

	(g)
Ascorbic acid	45
Nicotinic acid	36
Vitamin B_{12}	30
Ca-d-pantothenate	24
Thiamine HCl	6
Riboflavin	6
Folic acid	3
Pyridoxine	6
D-biotin	0.6
Menaphthone	0.6
Rovimix E500 (250mg E per g)	180
Rovimix A 500 (500,000 IU A per g)	11
Rovimix A500/D3 (100,000 IU D3 per g)	7

TABLE 5.3 DIETS

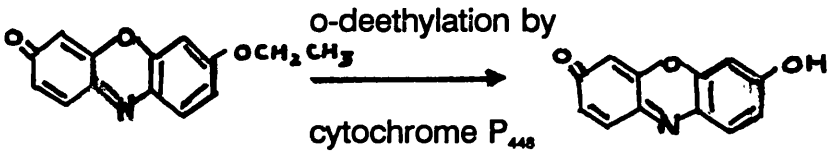
	% Composition (w/w)		
Corn oil	0	8	25
Corn starch	69	61	44
Casein	20	20	20
Vitamins	2	2	2
Solkafloc	5	5	5
Minerals	4	4	4
Energy (KJ/100g)	1513	1698	2063

Corn oil was estimated by GLC and found to contain 84% unsaturated fatty acids identified as 29% oleic (C18:1) and 55% linoleic (C18:2)

peroxidisability, expressed as the double bond index (DBI) value.
(iv) Assay of vitamin E concentration.

5.1.2.3 CYTOCHROME P_{448} ASSAY

Activity of cytochrome P_{448} has been studied using the highly specific substrate ethoxyresorufin. The o-deethylation of this substrate to resorufin (see equation below) by rat and hamster liver microsomes has been shown to be exclusively catalysed by cytochrome P_{448} (Burke & Mayer 1975).



7-ethoxyresorufin resorufin

The direct fluorimetric assay for O-deethylation of ethoxyresorufin described, had the advantages of being simple and very sensitive. It was performed in Dr De Matteis' laboratory at The Medical Research Council Toxicological Unit, Carshalton.

Microsomes were transported in dry ice and remained frozen. They were thawed and resuspended in 5 ml of 1.15 % KCl; 100 µl of resuspended pellet was diluted for protein estimation by the modified Lowry method.

Incubations were carried out at 25 °C in a fluorimeter cuvette containing 2.13 ml 50 mM Tris-HCl buffer with 25 mM $MgCl_2$, pH 7.5, 100 µl of microsomal suspension (0.3-0.4 mg protein ml^{-1}), 10 µl of ethoxyresorufin dissolved in DMSO (2 µM final conc.) and 10 µl NADPH in buffer (125 µM final conc.)

Reactions were started by adding NADPH and stirring. The progressive increase in fluoresence, resulting from deethylation of ethoxyresorufin to resorufin, was

recorded at an excitation wavelength of 522 nm and slit width 2.5 mm and an emission wavelength of 586 nm and slit width 5 mm, using a Perkin-Elmer LS5 I uminesence spectrophotometer.

Fluoresence was standardised using a standard block on the day of the assay. A standard curve for resorufin was prepared and the standard curve block reading was noted. The rate of ethoxyresorufin deethylation (i.e. increase in fluoresence per min) was calculated for each microsomal suspension and compared to the standard curve to obtain nmol resorufin formed per min. Correcting for the standard block and dilution factors and for mg of protein, the activities were expressed as pmol of resorufin formed per min per mg protein.

5.1.2.4 EXTRACTION AND SEPARATION OF MICROSOMAL PHOSPHOLIPIDS.

Microsomal pellets were resuspended in 5 ml 40 mM MOPS buffer pH 7.4. An aliquot was diluted for protein determination. 4 mg of microsomal protein were used for lipid extraction by a method based on that of Bligh and Dyer (1959).

5 ml methanol were added dropwise while vortexing to 1 ml of microsomal suspension containing 4 mg protein. The mixture was allowed to stand for 10 min at room temperature, after which 2.5 ml chloroform were added and vortex mixed. The resulting mixture was centrifuged at 2,500 rpm for 5 min to precipitate the protein, which was discarded. The supernatant was transferred to a graduated glass tube and 2.5 ml of chloroform and 2.5 ml water were added and mixed. The tube was then centrifuged at 2,500 rpm for 5 min to separate the aqueous and chloroform phases. The top aqueous phase was discarded. 200 μl methanol were added to the lipids, in the chloroform layer, to make the system homogenous. The extracted lipids were stored in sealed tubes under nitrogen at -20 °C until required. The chloroform layer was made up to or reduced down to exactly 5 ml, so that phospholipid could be expressed as per mg microsomal protein.

The mixture obtained at this stage contains triglycerides, free fatty acids and phospholipids as well as other contaminants such as inorganic phosphorus. It was therefore necessary to isolate the phospholipids to obtain a pure phospholipid preparation for fatty acid analyses (see below). Purification was carried out by a separation procedure using Bond Elut columns.

Separation of phospholipids using Bond Elut columns.

Bond Elut columns (size 3cc with Aminopropyl bonded phase) obtained from Analytichem International, were used to isolate triglycerides (TGs), free fatty acids (FFAs) and phospholipids (PLs) in high yield and purity (Kaluzny et al, 1985).

Columns were washed with 4 ml hexane. 0.5 ml of the chloroform samples were loaded and washed with 4 ml chloroform / isopropanol (2:1), which eluted the TGs. Washing with 4 ml of ether / acetone (98 : 2, v/v), removed FFAs. The final wash with 4 ml of methanol yielded phospholipids.

5.1.2.5 DETERMINATION OF MICROSOMAL PHOSPHOLIPID FATTY ACIDS.

Microsomal phospholipid fatty acid (PLFA) profiles were determined in order to be able to calculate a total double bond index for each microsomal sample. The **Double bond index** for a particular unsaturated fatty acid is equal to the percentage mass (which is equivalent to the area represented by each fatty acid, expressed as a percentage of the total fatty acids identified, obtained from gas liquid chromatography (GLC) analysis), multiplied by the number of double bonds in that fatty acid. This index was calculated for the major unsaturated fatty acids (C16:1, palmitoleic; C18:1, oleic; C18:2, linoleic; C18:3, linolenic and C20:4, arachidonic) in microsomal phospholipid and summated to give the total DBI for that particular sample. Total DBI thus reflects the number of potentially oxidizable double bonds and may be regarded as a crude measure of the peroxidizability of

the membrane, since other membrane factors such as vitamin E content also influence peroxidation. Decosohexanoic acid (C22:6) is also one of the major PLFAs in microsomes but it was not used in the DBI calculation because a standard was not available for GLC analysis.

Esterification and methylation of PLFAs in preparation for GLC.

Phospholipids eluted from the Bond Elut column were evaporated to dryness in a N_2 stream at about 40 °C. An ethanol and hexane mixture (1:1, v/v) was added (200 µl) and evaporated under N_2 to remove water. 1 ml boron trifluoride in methanol was added quickly under N_2 and the glass tubes were sealed, then heated at 90 °C for 1h. The methylated fatty acid esters were extracted into 5ml hexane / water (1 : 1) by vortex mixing and centrifugation. The top hexane layer was transferred to a clean tube and evaporated to dryness. Further 100 µl of hexane were added and again evaporated. Samples were redissolved in 100 µl cyclohexane and stored at -20°C, in sealed tubes under N_2.

The methylated fatty acids were subjected to GLC analysis to determine the percentage of the total area represented by each fatty acid. This information was used to calculate DBIs as described above.

Gas liquid chromatography of methylated phospholipid fatty acids.

Gas liquid chromatography was carried out using a 6ft. glass column of 0.25 inch outer diameter 2mm internal diameter in a Hewlett Packard 5840 A dual flame ionising chromatograph. The stationary phase was 15% polyethylene glycol succinate absorbed onto Gaschrom Q, the support phase. Column temperature was 175°C and the injector and detector temperature 180 °C with gas flows of nitrogen 50 ml min^{-1}, hydrogen 50 ml min^{-1} and air 600 ml min^{-1}. Retention times of the individual fatty acids to allow identification and tests of the linearity of detector response, were obtained using standard methylated fatty acids (Sigma Ltd). The

detector response was similar for all fatty acids detected. The relative quantities of individual fatty acids were calculated from measurments of the peak areas using a 5480 A gas chromatograph integrator. Major microsomal phospholipid fatty acids were identified up to and including arachidonic acid.

5.1.2.6 EXTRACTION AND ASSAY OF VITAMIN E.

Extraction and assay of vitamin E from hepatic microsomes of rats maintained on 0 %, 8 % or 25 % lipid diets were undertaken by Mr. Christopher Dark in Dr.D. Muller's laboratory at The Institute of Child Health, London. The method described, is a modification of the method described by Butriss and Diplock (1988).

Vitamin E was determined by high performance liquid chromatography (HPLC) with fluorimetric detection. Routinely, approximately 100 mg of tissue samples were homogenised in 1ml 75 % ethanol in water at 4 °C using a Polytron disintegrator at full speed for 30s. Hydrophobic lipid species were extracted into 1 ml cold HPLC grade hexane (Fisons PLC) by mixing for 60s. Hexane phase (10 μl) was injected onto a 25 cm, 5 mm silica direct phase column with an internal diameter of 4.9 mm (Jones Chromatography Ltd.) and eluted at 2 ml min^{-1} with hexane, containing 1 % methanol, which had been dried with 4 Angstrom molecular sieves. Fluoresence was monitered with a Perkin Elmer LS1 fluorimeter using an excitation cut off filter at 280 nm and a 310 nm emission filter. Concentrations of vitamin E were determined by direct comparision with external standards.

5.1.3 RESULTS AND DISCUSSION.

5.1.3.1 EFFECT OF DIETARY LIPID ON PLASMA BR AND *IN VITRO* LPO AND BRD ACTIVITIES.

TABLE 5.4 MEAN CHANGES IN BODY WEIGHT, LIVER WEIGHTS AND FOOD CONSUMPTION AFTER 14 DAYS ON DIFFERENT LIPID DIETS

	Corn oil in diet		
	0%	8%	25%
Change in body wt (g)	12.0 ± 4.0	29.0 ± 4.0	28.0 ± 4.0
Liver wt (%)	2.5 ± 0.1	2.9 ± 0.1	2.8 ± 0.04
Food consumption (g/rat/day)	17.9 ± 3.2	19.4 ± 1.7	15.3 ± 2.1

All values are means ± SEM for 8 animals in each group. Liver weight is expressed as a percentage of the body weight at the end of the experiment.

The mean weight of food consumed is expressed as g/rat/day.

There were significant differences between the 0% and 25% lipid groups for body weight ($p < 0.01$) and liver weight ($P<0.001$). No significant difference between 8% and 25% lipid groups.

Food consumption by rats on the three diets was similar (Table 5.4). They consumed 15-20g per rat per day. All rats maintained body weight over the two week dietary period; the mean increase in
body weight was similar for rats receiving the 8% and the 25% lipid but less for the lipid free group (Table 5.4). The mean liver weights, expressed as percentage of body weight, were similar in all three groups.

Livers were removed and fixed in formalin. Sections were cut and stained with haemotoxylin and eosin and also with oil red. All rats showed normal liver histology irrespective of the diet indicating that there was no accumulation of triglycerides.

The high lipid diet produced a significant mean decrease in plasma BR of 46.1 ± 3.6 % (n = 8) over the 2 week dietary period in all rats. In contrast, rats on the lipid free diet, showed a significant increase in plasma BR of 62.5 ± 25.8% (n = 6), while rats on the 8 % lipid diet showed 10.0 ± 5.1 % (n = 6) decrease in plasma BR (Fig 5.1).

Significantly higher enzymatically initiated BRD and LPO activities were evident during 10 min incubation of microsomes from rats fed the high lipid diet; 8 % lipid diet produced lower LPO and BRD activities while the lipid free diet did not produce detectable LPO or BRD activities even over 20 min incubation (Fig 5.2, 20 min data not shown). Others have reported an inhibition of LPO in rats fed a lipid free diet (Hammer & Wills, 1978, Janssen & Schenkman, 1975).

These results suggest that the increase in plasma BR in rats on the lipid free diet could be due to a decrease in the oxidative destruction of BR as a result of diminished LPO *in vivo*. The decrease in plasma BR in rats on the high lipid diet could be due to increased LPO *in vivo* causing greater oxidative destruction of BR.

In order to explore the mechanisms underlying these diet modulated effects, LPO was initiated non-enzymatically with 0.2 mM ascorbate in heat-treated microsomes

(83 °C for 5 min) from the dietary groups. If the high lipid diet promotes greater LPO activity by induction of microsomal enzymes then heat-treatment of microsomes would be expected to destroy these enzymes. Similarly, if the decreased BRD and LPO activities in the lipid free group were due to changes in other enzymatic pathways, these would also be abolished. The observed differences in LPO and BRD activities between the groups of microsomes, as described for both NADPH and ascorbic acid initiated systems, would therefore be eliminated. However, if the basis of these differences is not enzymatic, then they would persist in heat denatured microsomes, as indeed is shown in Fig 5.3 which shows similar findings to those presented in Fig 5.2. This suggests that LPO and BRD must be influenced by other factors such as the lipid composition or the lipid antioxidant concentration in microsomes from the 3 dietary groups.

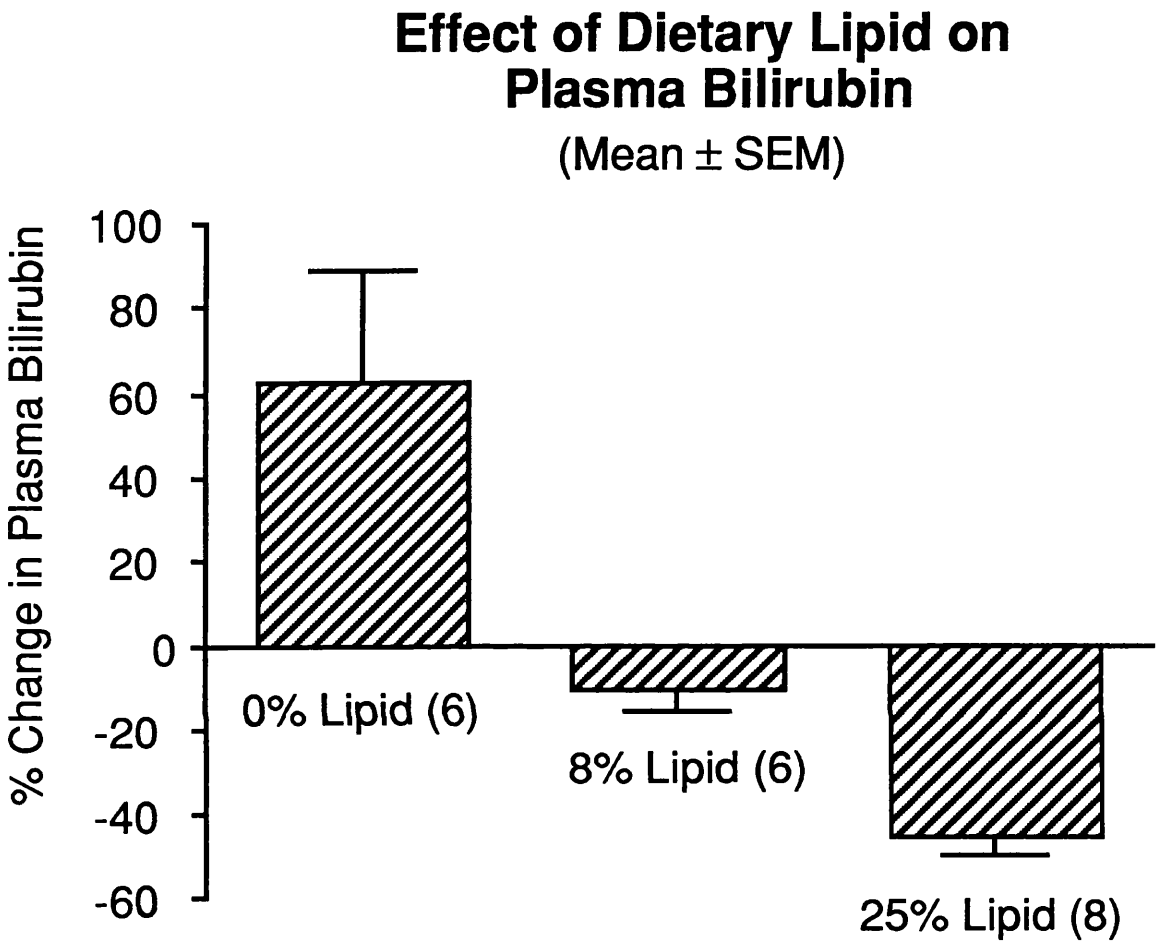
5.1.3.2 EFFECT OF DIETARY LIPID ON MICROSOMAL CYTOCHROME P_{448} ACTIVITY.

Figure 5.4 shows that although the cytochrome P_{448} activity is lower in microsomes from the lipid free group compared with the 25 % lipid group the difference between them was not significant. Activities in all the synthetic diet groups were lower than in the control group and may be due to other differences between the diets. These results agree with the above findings that the high LPO activity in the 25 % lipid group is not due to induction of a specific microsomal enzyme and that the lack of LPO activity in the lipid free group is also not due to an enzymatically mediated mechanism. Inducers of cytochrome P_{448} have been shown to decrease plasma BR concentration (De Matteis et al, 1989); however, the results presented here show that the dietary effects do not involve the same mechanism.

5.1.3.3 EFFECT OF DIETARY LIPID ON THE MICROSOMAL PHOSPHOLIPID FATTY ACID DOUBLE BOND INDEX.

The percentage composition of the major microsomal PLFA from rats maintained

Fig 5.1

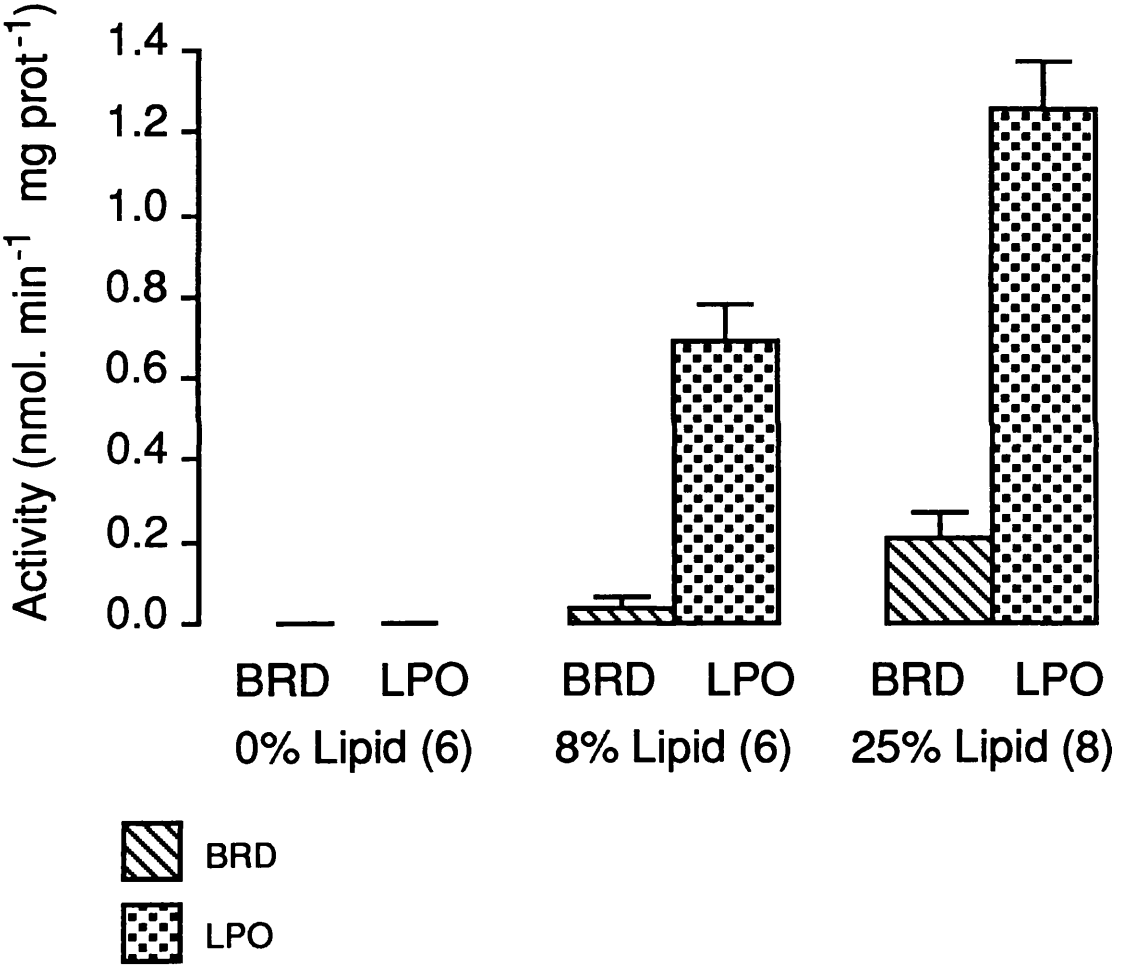


Gunn rats were maintained for 2 weeks on diets containing 0%, 8% and 25% corn oil. Blood samples were taken at the beginning and end of the experiment and the change in plasma bilirubin expressed as a percentage of the initial value. The numbers in each group are shown in parenthesis.

Fig 5.2

Effect of Dietary Lipid on Enzymatic Bilirubin Degradation (BRD) and Lipid Peroxidation (LPO)

Mean ± SEM

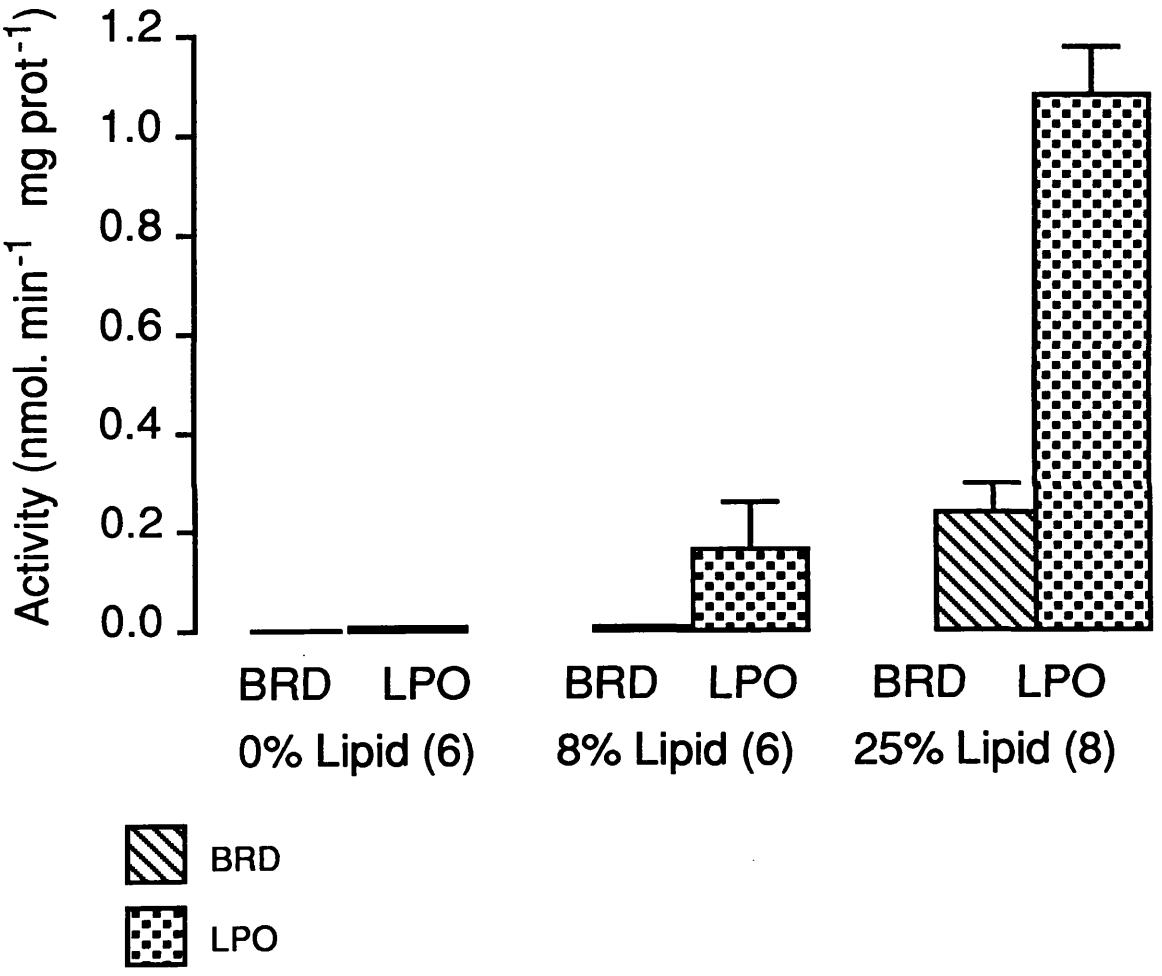


Hepatic microsomes were prepared from rats maintained on 3 different diets, as described previously and their enzymatically initiated LPO and BRD activities were determined *in vitro* over 10 min.

Fig 5.3

Non-Enzymatic Bilirubin Degradation (BRD) and Lipid Peroxidation (LPO) in Heat-treated Microsomes

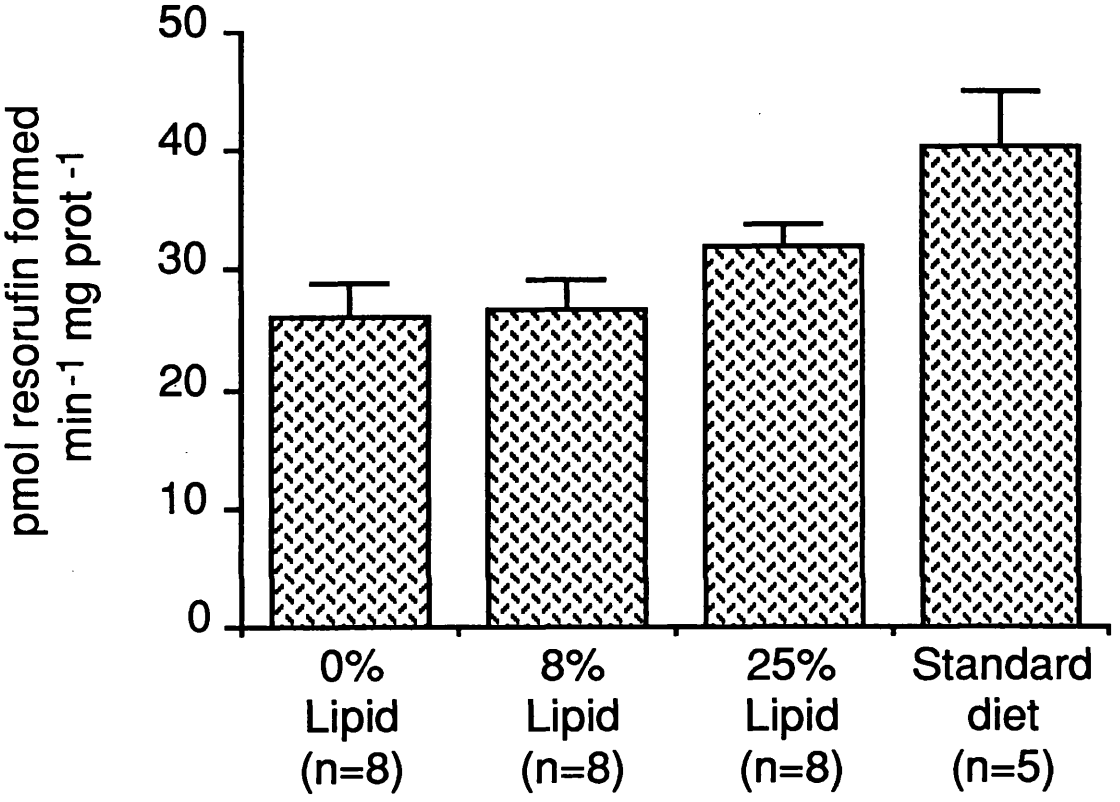
Mean ± SEM



Hepatic microsomes from rats maintained on 3 different diets were subjected to heat treatment at 83 °C for 5 min. LPO was initiated non-enzymatically with 0.2 mM ascorbic acid and LPO and BRD activities were determined *in vitro* over 10 min.

Fig 5.4

Cytochrome P_{448} Activity in Microsomes from the Three Dietary Groups



7-ethoxyresorufin O-deethylase activity was estimated as an assay for cytochrome P_{448} in hepatic microsomes from rats fed diets containing 0%, 8% and 25% corn oil for 2 weeks. No significant differences were found but these activities were less than those obtained with microsomes from rats fed a standard diet.

on different lipid diets and standard diet are shown in Table 5.5.I. The most significant change was observed in the percentage of arachidonic acid which was significantly higher in the 25% lipid group compared with the lipid free group. Table

5.5.II shows individual and total DBI values for PLFA from microsomes from each group of animals on the different lipid diets.

Figure 5.5 shows the total DBI for hepatic microsomal PLFA from rats maintained on 0 %, 8 %, and 25 % corn oil diets. The DBI values for the 8 % and 25 % groups were greater and significantly different from the 0 % group ($p < 0.05$). The DBI for the 0 % lipid group was less than that for the group on the standard diet. The lack of significant difference between the DBI values in the 8 % and 25 % lipid groups, suggests that there is little difference in the degree of unsaturation of the microsomal membrane fatty acids obtained with these 2 diets.

One can conclude that the corn oil containing diets altered the PLFA composition of the liver endoplasmic reticulum in such a way that their peroxidizability was greater than that of the lipid free diet. This suggests that the fatty acid composition of the liver endoplasmic reticulum is an important factor controlling the rate and extent of lipid peroxidation *in vitro*, and possibly *in vivo*. However, care is needed in using the DBI as the sole index of LPO, because other dietary and endogenous components may also be important; for example, a deficiency in the antioxidants vitamin E or glutathione would result in increased LPO.

5.1.3.4 CONCENTRATION OF VITAMIN E IN MICROSOMES FROM THE 3 DIETARY GROUPS.

Table 5.6 shows the vitamin E concentration in the different diets and in microsomes from the 3 dietary groups expressed as μg vitamin E per g wet wt pellet, per mg phospholipid or per mg protein in pellet. All groups on the synthetic diets, had greater microsomal vitamin E concentrations than the group on the

TABLE 5.5

I. PERCENTAGE COMPOSITION OF MICROSOMAL PHOSPHOLIPID FATTY ACIDS UP TO AND INCLUDING ARACHIDONIC ACID (MEAN ± SEM)

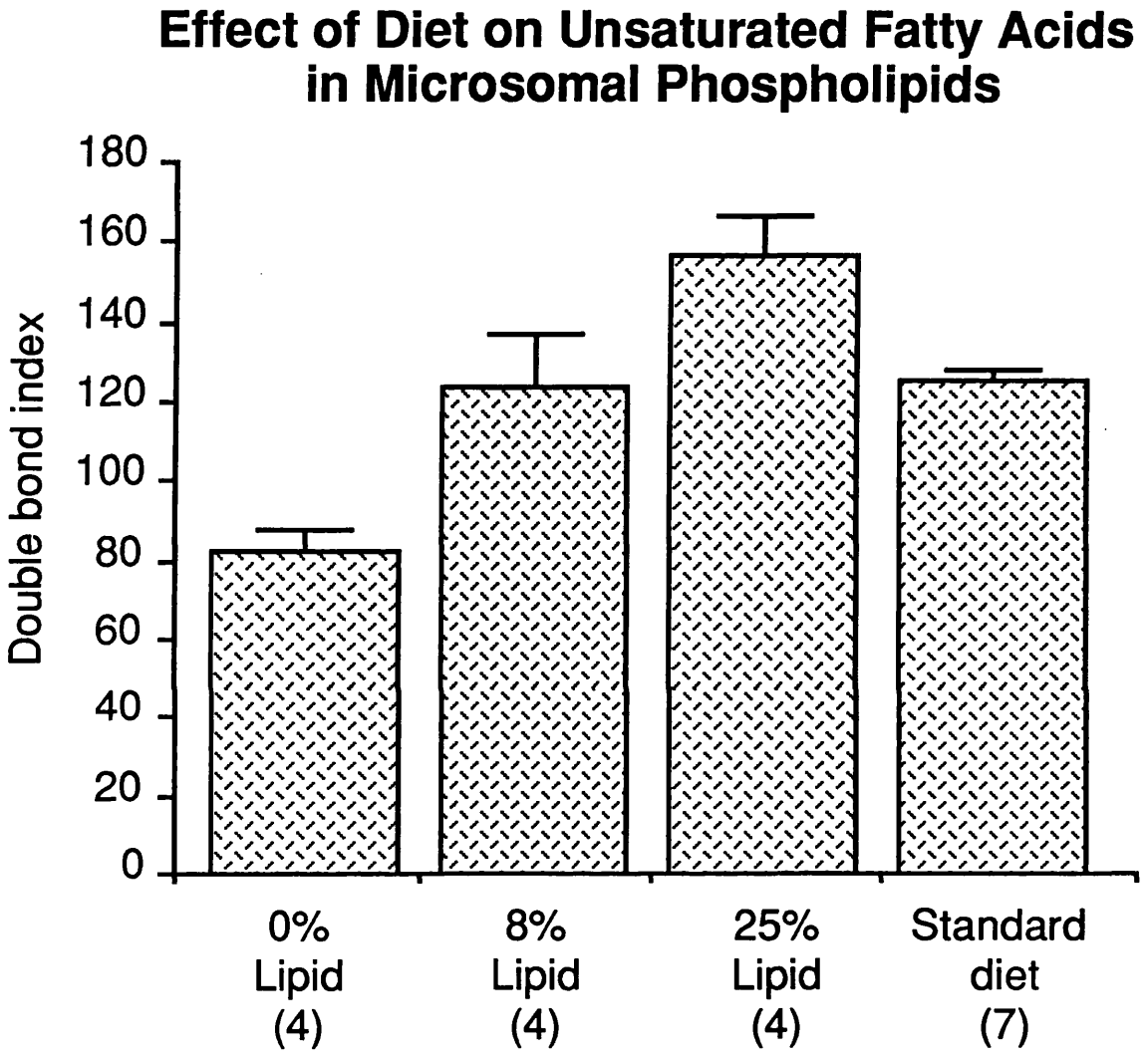
Diet (% lipid)	n	C16:0	C18:0	C16:1	C18:1	C18:2	C18:3	C20:4
0	3	26.1 ±2.6	33.6 ±1.4	3.5 ±0.6	18.7 ±3.3	7.8 ±0.5	0.5	10.9 ±1.5
8	4	16.3 ±1.3	40.0 ±3.3	-	9.8 ±1.0	10.8 ±1.0	-	23.2 ±3.1
25	4	11.6 ±3.1	34.8 ±3.2	-	10.4 ±1.0	8.4 ±0.4	4.4	30.2 ±3.0
Standard	7	24.2 ±0.8	29.1 ±0.9	0.89 ±0.2	9.13 ±0.4	16.8 ±0.5	4.47 ±0.2	17.04 ±0.7

II. DOUBLE BOND INDEX FOR INDIVIDUAL FATTY ACIDS AND TOTAL DBIs (MEAN ± SEM)

Diet (% lipid)	n	C16:1	C18:1	C18:2	C18:3	C20:4	**Total DBI**
0	3	3.5 ±0.6	18.7 ±3.3	15.6 ±1.0	4.8	43.6 ±6.0	**81.6 ±5.9**
8	4	-	9.8 ±1.2	21.6 ±2.0	-	92.8 ±12.4	**124.1 ±12.9**
25	4	-	10.4 ±1.0	16.8 ±0.8	13.2	120.8 ±12.0	**156.8 ±10.0**
Standard	7	0.89 ±0.2	9.13 ±0.4	33.6 ±1.0	13.41 ±0.6	68.17 ±2.8	**125.3 ±2.7**

Compared with the 0% lipid group, the total DBI values were significantly higher in the 8% lipid group ($p<0.05$), 25% lipid group ($p<0.01$) and standard diet group ($p<0.05$)

Fig 5.5



The double bond index was determined in hepatic microsomes from rats for 0%, 8% and 25% corn oil in the diet for 2 weeks as described in section 5.1.2. A significant difference between the indices for the 0% and 25% lipid groups was observed ($p < 0.001$).

TABLE 5.6 VITAMIN E IN MICROSOMES FROM RATS MAINTAINED ON DIFFERENT DIETS

Dietary Lipid %	Calculated Vitamin E mg/kg diet	Microsomal Vitamin E (mean ± SEM) μg/g wet wt Pellet	μg/mg Phospholipid	μg/mg Protein
0	76.3	* 23.8 ± 1.1 (4)	0.91 ± 0.08 (4)	0.38 ± 0.01 (4)
8	81.2	19.5 ± 4.0 (4)	0.85 ± 0.07 (4)	0.33 ± 0.05 (4)
25	91.5	* 13.2 ± 4.7 (4)	0.75 ± 0.11 (3)	0.26 ± 0.07 (4)
Standard diet	68.3	6.4 ± 2.7 (3)	0.34 ± 0.11 (3)	-

Numbers in parenthesis represent the number of animals in each group.

* $p < 0.05$

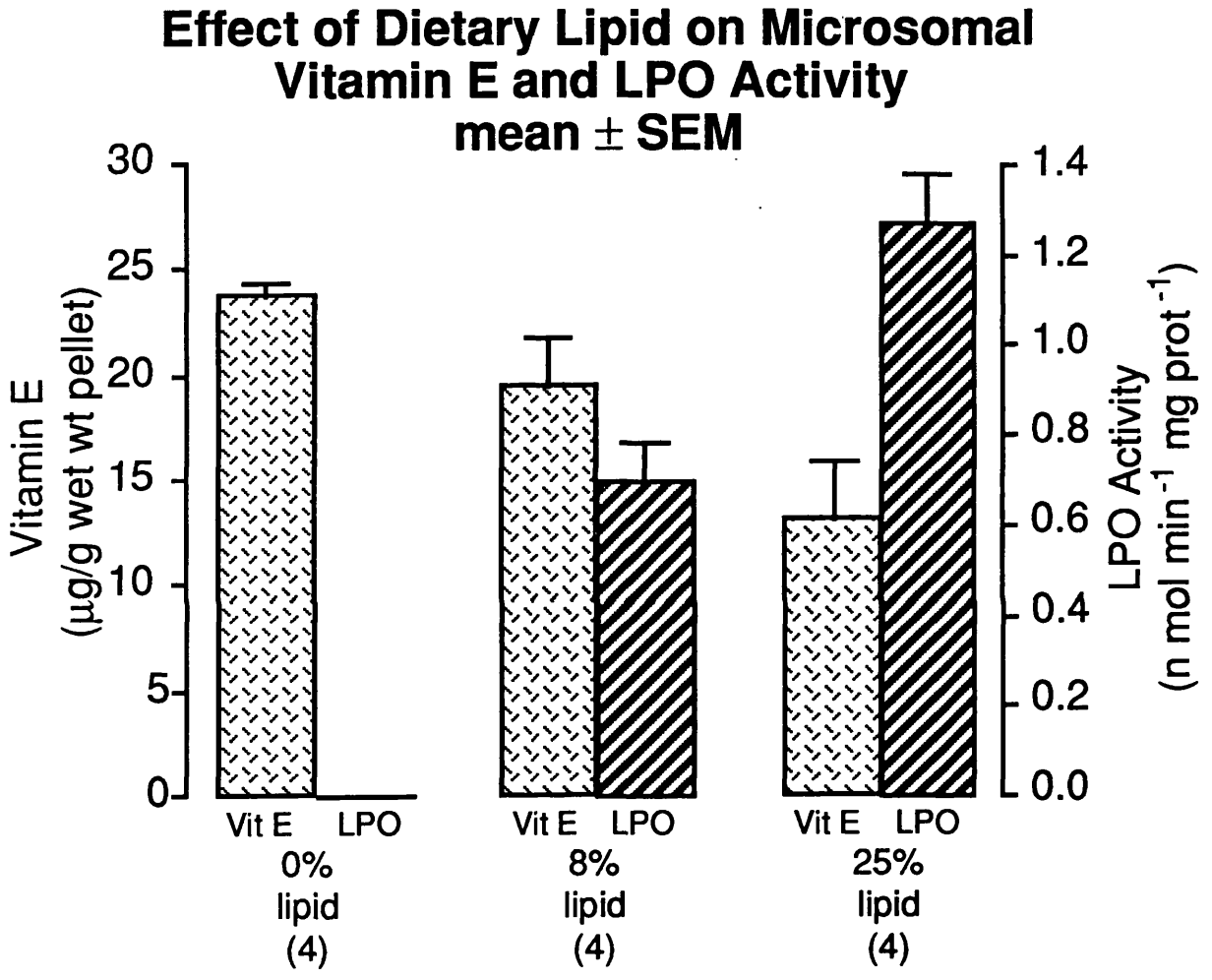
standard diet.

Whichever way the vitamin E concentration was expressed, the highest values were found in the rats on the 0 % lipid diet; slightly lower values were found in those on the 8 % lipid diet, while the microsomes from the 25 % lipid diet had the lowest vitamin E concentration. When these results were expressed as μg vitamin E per mg microsomal phospholipid or per mg protein, there was no significant difference between the results for rats fed the lipid free and high lipid diets. However, there was a significant difference between the results of these two groups when expressed per wet weight of pellet ($p < 0.05$). The microsomal vitamin E concentration was inversely related to the dietary intake of vitamin E (Table 5.6).

One could argue that the higher microsomal vitamin E concentration in the lipid free group is responsible for inhibiting LPO in this group and that an *in vivo* mechanism is responsible for conserving vitamin E. This mechanism would presumably be associated with the dietary lipid content. An alternative explanation is that the lower vitamin E concentration in the 25 % lipid group compared with the 0 % lipid group, results from vitamin E being consumed to a greater extent in the former group due to the higher LPO activity (Fig 5.6).

It is unlikely that vitamin E is the sole factor responsible for regulating LPO. *In vivo*, there is most likely to be a balance between factors that initiate and propagate LPO and antioxidants that terminate the process. Whether vitamin E is inhibiting the initiation of peroxidation or whether it becomes effective at a later stage, cannot be resolved from these experiments.

Fig 5.6



Hepatic microsomal vitamin E (expressed as mg/kg wet weight pellet) and enzymatic LPO activities were determined in rats fed 0%, 8% and 25% lipid diets. In the 0% lipid group the high vitamin E concentration was associated with minimal LPO activity while in the 25% lipid group the vitamin E conc was significantly lower ($p < 0.05$) and the LPO activity was higher ($p < 0.001$).

5.2 EFFECT OF VITAMIN E ON PLASMA BILIRUBIN CONCENTRATION AND MICROSOMAL LIPID PEROXIDATION AND BILIRUBIN DEGRADATION.

5.2.1 INTRODUCTION

The dietary experiments had suggested that variations in LPO could be responsible for the changes in plasma bilirubin. To investigate this finding further, Gunn rats were injected with the antioxidant vitamin E, which is known to abolish LPO, in order to observe its effect on plasma bilirubin. Vitamin E studies were performed using rats from the old established colony and a 'New colony'.

5.2.2 New Colony of rats

The Royal Free Hospital School of Medicine colony of Gunn rats was established in the 1960s. It was noticed that litter sizes in the colony had markedly decreased and fertility was low. In order to maintain the colony and obtain a better yield, it became necessary to cross breed with healthy Wistars and obtain a 'New Colony'. Males from the old colony of Gunn rats were crossed once with female Wistar rats. The female offspring (heterozygotes) were then crossed with male Gunn rats from the original colony and the jaundiced off spring were inbred to obtain the new colony.

5.2.3 Study 1 - Standard diet

Experimental

A solution of vitamin E (Sigma) (50 mg ml^{-1}) was prepared for injection by dissolving it in one volume ethanol and nine volumes 16 % (v/v) Tween 80, which had been diluted in 0.9 % (w/v) NaCl (Corongiu et al 1985).

Eight male Gunn rats (old colony, 225-326 g) were maintained on a standard diet and given water ad libitum. Rats were injected intra peritoneally (i.p) with vitamin E (110 mg per kg body weight). Three rats were sacrificed 24h and 48 h after injection. The other 2 rats were sacrificed after 72 h. Initial and final plasma BR concentrations were determined. Hepatic microsomal LPO and BRD activities were determined.

In a second study, the effect of vitamin E on plasma BR concentration was investigated in 8 rats from the new colony. Rats were injected with vitamin E (110 mg kg^{-1} body wt). Blood samples were taken through the tail vein under halothane anaesthesia on days 0, 4 and 8. On day 4, 4 of the rats were reinjected with vitamin E.

Results and Discussion

A preliminary study in 3 rats, showed that i.p. administration of the ethanol / Tween 80 vehicle had no haemolytic effect; the plasma BR concentration did not change over 48 h after injection (data not shown). A significant change in plasma BR was arbitarily chosen as 10 μM in order to account for possible day to day variations.

Table 5.7 shows that in all animals vitamin E completely inhibited microsomal LPO and BRD activities regardless of the time of sacrifice. Inhibition of BRD *in vivo* was expected to result in an increase in plasma BR concentration. However in 4 out of 8 rats there was a significant decrease in plasma BR concentration. Plasma BR concentration in two other animals, remained more or less constant (+ 3 %, - 2 %) up to 3 days after injection and in only two rats the plasma bilirubin increased. Table 5.8 shows the results obtained for the 8 rats from the new colony that were injected with vitamin E. Four days after vitamin E injection, 5 rats showed no significant change in plasma BR, 1 showed a decrease and 2 showed an increase. The marked change in

TABLE 5.7 EFFECT OF VITAMIN E ON PLASMA BR AND HEPATIC MICROSOMAL LPO AND BRD ACTIVITIES OVER 72h (old colony)

Time after vitamin E (h)	n	Plasma BR (% Change)	MICROSOMAL ACTIVITY (nmol min^{-1} mg $prot^{-1}$) LPO	BRD
24	3	+ 17.4 - 13.4 + 3.1	0.00	0.00
48	3	+ 7.6 - 25.7 - 2.1	0.02	0.00
72	2	- 31.9 - 19.7	0.00	0.00

TABLE 5.8 EFFECTS OF VITAMIN E ON PLASMA BILRUBIN (NEW COLONY)

	n	Day of injection	Plasma BR Conc (μM) Day 0	Day 4	Day 8
GROUP I					
1 dose of Vit. E (110mg kg body wt^{-1})	4	0	120	122	119
			105	95	101
			109	100	111
			71	98	91
GROUP II					
2 doses of Vit. E	4	0 & 4	107	114	122
			140	108	131
			106	97	108
			105	207	145

BR seen in 1 of these animals was associated with a considerable loss of weight which suggests that factors other than vitamin E may have been responsible; this rat will not be considered in further analysis of the data. In the group of rats that did not receive a second dose of vitamin E, the plasma BR on the eighth day was not significantly different from that on day 0 in 3 rats and, in the fourth rat the concentration was increased but was similar to that on the fourth day. The second dose of vitamin E did not appear to have a significant effect; the day 8 plasma BR was slightly raised compared with the initial level in 1 rat and in another rat the level was higher than at 4 days, otherwise there were no changes. The antioxidant effect of vitamin E which is evident from the complete inhibition of microsomal LPO activity in vitamin E treated rats could have been responsible for the elevated plasma BR observed in a minority of animals, but the evidence is inconclusive.

5.2.4 Study 2: Effect of vitamin E on plasma BR and microsomal LPO and BRD activities in rats maintained on a 25 % lipid diet.

Introduction

In the previous experiments, it was anticipated that the administration of vitamin E would result is a reduction in LPO and that this would probably cause a rise in plasma bilirubin such as was observed when microsomal LPO activity was abolished by the use of a lipid free diet. The expected rise in plasma BR did not, however, occur. A possible explanation is that the mechanisms of action of vitamin E and dietary lipid in modulating microsomal LPO are very different. While vitamin E inhibits LPO at the terminal stage of the chain reaction (Chapter 2), a lipid free diet is likely to decrease LPO by influencing the unsaturation of membrane phospholipid fatty acids.

If BRD is regulated by the lipid radicals that arise during the propagation stage of LPO, then vitamin E would be unlikely to influence plasma BR concentration. In order to explore this matter further, the following study investigated the effect of

vitamin E on plasma BR in rats fed a high lipid diet which has been shown to increase LPO.

5.2.4.1 Study 2a

Experimental (New colony)

Two groups of male Gunn rats (300-365 g) were maintained on a 25 % lipid diet throughout the study. The experimental group (n = 5) was injected i.p. with vitamin E (110 mg per kg body wt) on day 14, while the control group (n = 4) received an equivalent amount of the Tween 80 vehicle. Rats were sacrificed on day 17 and hepatic microsomes were prepared for LPO and BRD determinations. Tail vein blood samples were taken at the start of the diet and on days 14 and 17 for plasma BR determinations.

Results and Discussion

In contrast to previous findings, only 4 out of 9 rats (44 %) produced a decrease in plasma BR after 2 weeks on a high lipid diet; the other rats did not respond (Table 5.9). This poor response to the diet may be explained by the fact that rats were used from the new colony.

Three days after vitamin E treatment, two rats showed a significant increase in plasma bilirubin concentration. One showed a decrease while the others showed no significant changes. In the control group, the plasma BR in the three rats that responded to diet, did not produce consistant changes after the vehicle injection. Two rats had an increase in BR concentration while one had a slight decrease; however, all three plasma BR concentrations continued to be markedly less than the initial value as would be expected for rats maintained on the high lipid diet. The one rat that did not respond to diet in the control group, continued to be unaffected. Lipid peroxidation and bilirubin degradation activities were completely

TABLE 5.9 EFFECT OF VITAMIN E (110 mg kg body wt^{-1}) ON PLASMA BR AND LPO AND BRD ACTIVITIES IN RATS ON A 25% CORN OIL DIET

Colony + Treatment	Plasma BR conc. (μM)				Hepatic microsomal activity (nmol min^{-1} mg $prot^{-1}$)	
	Intial	After 2 weeks on diet	3 or 4(*) days after Vit E	7 days and 2 doses of Vit E	LPO	BRD
New	158	127	158	-		
+	97	103	89	-		
Vit E	106	110	131	-	0.03	0
(n = 5)	112	116	118	-	±	
	110	112	106	-	0.02	
New	188	162	151	-		
+	172	93	121	-	1.41	0.31
vehicle	158	98	121	-	±	±
(n = 4)	153	156	152	-	0.05	0.03
Old	129	92	- *	111		
+	125	97	116	-	0.01	0
Vit E	122	90	119	-	±	
(n = 4)	119	93	-	132	0.01	
Old	155	99	144 *	-		
+	128	96	-	132	1.32	0.36
vehicle	122	96	-	116	±	±
(n = 4)	140	95	127	-	0.13	0.05

Vitamin E was injected on days 14, or 14 and 17, after start of diet. LPO and BRD activities represent Mean ± SEM of all animals.

There is a significant difference in LPO and BRD activities between vitamin E and vehicle injected rats for both old and new colonies ($P < 0.005$).

inhibited in the vitamin E treated group whereas the vehicle had no inhibitory effect.

5.2.4.2 Study 2 b

Because of the variation in response of the new colony rats to the high lipid diet, this study was repeated with some rats remaining from the old colony.

Experimental (old colony)

Two groups of male Gunn rats (250-300 g) from the old colony were maintained on a 25 % lipid diet throughout the study. On day 14, the rats were either injected with vitamin E (110 mg per kg body wt), (n = 4), or with the vehicle, (n = 4). On day 18, two rats from each group were sacrificed. The other two rats were reinjected with either vitamin E or vehicle and sacrificed 3 days later. Tail vein blood samples were taken at the start of the diet and on days 14, 18 and 21 for measurement of plasma BR concentration. Hepatic microsomal LPO and BRD activities were determined at the time of sacrifice.

Vitamin E was administered twice in this dietary study in order to observe the effect of prolonged exposure of the antioxidant on plasma BR. Since no further animals remained from the old colony, it was not possible to extend this part of the study further.

Results

All rats responded to the high lipid diet by a decrease in plasma BR concentration (in agreement to the previous findings section 5.1.3). Mean percentage decrease in concentration was 26.44 ± 1.68 %, n = 9. Vitamin E injection produced significant increases in plasma BR in each of 4 rats whether one or two doses of vitamin E were given. Significant increases in plasma BR were also obtained in rats

receiving the vehicle injection (Table 5.9).

Vitamin E completely inhibited NADPH initiated microsomal LPO and BRD activities, while the vehicle had no effect.

5.2.5 DISCUSSION

The fact that in the high lipid diet, the vehicle itself produced an increase in plasma BR in all animals in the old colony and two out of four rats in the new colony indicates that under these conditions Tween 80 has a hyperbilirubinaemic effect. This was a surprising finding since the vehicle failed to produce changes in BR concentration in the preliminary studies with the standard diet and the microsomal LPO and BRD activities were normal in all cases.

As an antioxidant, vitamin E would be expected to inhibit LPO *in vivo* and in accordance with the previously reported association between LPO and BRD (Chapter 3), it was anticipated that plasma BR would increase after treatment with vitamin E. In rats from the old colony fed the high lipid diet, the administration of vitamin E did cause a rise in plasma BR in all animals. However, this rise was no higher than that obtained when the Tween vehicle was injected so that it is not possible to conclude that vitamin E has caused the rise in plasma BR.

With rats from the new colony the results were more variable (Table 5.9) in both the vitamin E treated and control groups, even in a prolonged study (Table 5.8). When Gunn rats fed the standard diet were dosed with vitamin E, their changes in plasma BR were also variable.

As vitamin E is a lipid soluble antioxidant, it would be expected to accumulate in lipids and inhibit their peroxidation. Indeed vitamin E treated animals showed a complete inhibition of microsomal LPO and BRD activities while the vehicle had no effect in rats from both old and new colonies.

IN CONCLUSION, there is no convincing evidence from these experiments that vitamin E per se has a direct influence on overall bilirubin metabolism *in vivo*, although it does have a complete inhibitory effect on microsomal LPO and BRD activities.

The earlier off spring from new colony may have inherited factors from the healthy Wistars that made them resistant to the dietary effects. This is evident in the finding that the previous 100 % response to diet with the old colony was reduced to 50 % with the new colony. Exactly what these inherited resistant factors may be due to has not been investigated.

5.3 DBI DETERMINATION OF HEPATIC MICROSOMES FROM VITAMIN E TREATED AND CONTROL RATS.

The susceptibility of a membrane to peroxidation can be altered by changing the lipid composition of that membrane as has been demonstrated in the dietary lipid study. It is now well known that the ratio of phospholipid to cholesterol, the type of phospholipid present and the fatty acid composition all contribute to the structural integrity of the membrane. Any change in these variables, as occurs during tissue damage, renders the tissue more susceptible to oxidant stress (reviewed by Halliwell & Gutteridge, 1989).

It has been postulated, as a result of experiments using liposomes and with cell culture studies, that vitamin E in high concentrations may protect against lipid peroxidation by modifying membrane structure (reviewed by Halliwell & Gutteridge 1989). In order to demonstrate whether the decrease in microsomal LPO by vitamin E was the result of a loss of peroxidizability of the microsomal phospholipid fatty acids, DBIs were determined in vitamin E treated rat liver microsomes as previously described.

Results in Table 5.10 show that there was no difference in the hepatic microsomal

PLFA double bond index between vitamin E injected and control rats (receiving the vehicle injection) in the new colony. This suggests that, unlike the previous dietary lipid study (Fig 5.5) where an inhibition of LPO was associated with a decrease in the total DBI, the mechanism by which vitamin E inhibits LPO does not involve decreasing the peroxidizability of microsomal PLFA. A more likely mechanism could be that the vitamin E in the microsomal membrane scavenges the propagating lipid peroxy and alkoxy radicals, by donating labile hydrogen and thus terminates the chain reaction of peroxidation.

This hypothesis does not unfortunately hold for the old colony rats which had similar values for total DBI in the treated group as in the new colony, but had lower values in the control group ($p < 0.05$). Whether a strain difference was responsible for differences in vitamin E incorporation in membranes, resulting in a lower endogenous concentration of membrane vitamin E in the old colony, requires further investigation.

5.4 EFFECT OF ASCORBIC ACID ON PLASMA BILIRUBIN CONCENTRATION AND HEPATIC MICROSOMAL LPO AND BRD.

5.4.1 INTRODUCTION

Studies with the lipid soluble antioxidant vitamin E, showed that it inhibits LPO without the expected elevation in plasma BR concentration in Gunn rats. If LPO is the main route for BR degradation in the Gunn rat under normal dietary conditions, it is highly likely that the site of metabolism is in the liver endoplasmic reticulum, a membrane rich in peroxidisable fatty acids with the required reducing equivalents. However, peroxidation can take place in other parts of the body and with molecules other than lipids. The resultant free radical intermediates could then react with unconjugated BR at sites other than the liver.

Antioxidant protection is also provided by water soluble antioxidants *in vivo*.

TABLE 5.10 DOUBLE BOND INDEX IN HEPATIC MICROSOMES, 3 DAYS AFTER I.P. VEHICLE OR VITAMIN E (110 mg kg^{-1}) IN GUNN RATS RECEIVING STANDARD (S) AND 25% LIPID DIETS (L)

	Diet	n	Total double bond index
Old colony: - no injection	S	7	124.2 ± 2.7
New colony: - Vitamin E	S	5	119.6 ± 10.2
New colony: - Vitamin E	L	4	159.7 ± 5.1
- Vehicle	L	4	157.0 ± 5.6
Old colony: - Vitamin E	L	4	157.4 ± 3.1
- Vehicle	L	4	135.5 ± 8.7

Results are expressed as mean ± SEM

Differences in double bond index between control and vitamin E injected groups were not significant for the old or the new colony of rats. A significantly higher DBI. was observed for vitamin E injected rats maintained on 25% lipid diet compared with standard diets ($p < 0.02$, new colony).

Ascorbic acid has been suggested as an antioxidant of great physiological importance (Frei et al, 1988; Frei et al, 1989). It was therefore necessary to observe the effect of an aqueous antioxidant on plasma BR concentration in order to obtain further insight into radical mediated BRD *in vivo*. In addition, vitamin C has a well known synergistic effect on vitamin E (Niki, 1987b) and may have an anti-oxidant effect via this vitamin. A combination of vitamins E and C would be expected to prevent oxidation in both lipid and aqueous environments.

5.4.2 EXPERIMENTAL

All studies with ascorbic acid were in Gunn rats from the new colony. Groups of male Gunn rats (315-390 g) were injected i.p daily for 3 days with either saline (0.9 % w/v, NaCl, n = 3), or ascorbic acid in saline in doses of 180 mg per kg body wt (n = 4), 375 mg per kg body wt (n = 8) and 750 mg per kg body wt (n = 3). A group of 3 rats were injected with vitamin E (110 mg per kg body wt) on day 0; they also received 3 doses of vitamin C on days 0, 1, and 2 (750 mg per kg body wt).

Blood samples (0.75 ml) were taken on days 0 and 3 via the tail vein under halothane anaesthesia. Body weights were monitored. On day 3, animals receiving the low and intermediate doses of vitamin C were sacrificed. Hepatic microsomes were prepared.

In the saline group, the group receiving the highest dose of vitamin C and the group receiving combined doses of vitamins E and C, blood samples were taken repeatedly for a further 5 days after the last dose of vitamin C. Plasma BR concentrations were determined and expressed as percentage change from day 0; a significant change was arbitarily chosen as 10 % in order to account for possible day to day variations. Hepatic microsomes were prepared.

5.4.3 RESULTS AND DISCUSSION (Table 5.11)

There were no marked changes in plasma BR concentration when saline or ascorbic acid at a dose of 180 mg per kg body wt were administered, except in one case where an increase was observed. When the intermediate dose was administered, 50 % of the rats responded with an increase in plasma BR concentration while in the remainder there was no effect. This finding is reminiscent of the dietary vitamin E treated experiments with the new colony of rats in which the responses to treatment was also variable. At the highest dose, ascorbic acid produced very high increases in plasma BR concentration in 2 out of 3 animals. The overall effect of ascorbic acid and vitamin E was to increase the plasma BR concentration but not to the same extent as that seen with ascorbic acid alone.

In the prolonged study, plasma BR concentration showed a rapid drop (in 2 out of 3 rats) after vitamin C withdrawal; one animal died (Fig 5.7). The group of rats which had received both vitamins C and E showed a more gradual drop in plasma BR. The saline injected group also had a gradual fall in plasma BR after 3 days which might be due to repeated bleeding.

All animals lost weight in 3 days; however, the loss was not more than 10g in the saline and low dose ascorbate groups (Table 5.11). The weight loss was proportional to the dose of ascorbic acid administered and the increase in plasma BR, which suggests a possible toxic effect of ascorbic acid. In the intermediate dose group, both the responders and non responders lost weight.

Microsomal LPO activity appears to be slightly lower in the ascorbic acid treated groups which could be due to a synergistic effect between vitamin C and endogenous vitamin E. Vitamin E continued to inhibit LPO and BRD activities when administered with ascorbic acid even after 8 days.

It could be argued that the raised BR concentration is a consequence of the

TABLE 5.11 EFFECT OF I.P. ASCORBIC ACID FOR 3 DAYS ON PLASMA BR AND MICROSOMAL LPO AND BRD ACTIVITIES

I.P. Injection	% change in plasma BR after 3 doses ascorbic acid or saline	Change in body weight (g)	Activity (nmol min^{-1} mg $prot^{-1}$)	
			LPO	BRD
Saline* [a]	- 11	- 4	1.52 *	0.42
	- 6	- 3	1.61	0.34
	0	- 2	1.71	0.34
Ascorbic acid 180 mg kg^{-1}	- 2	- 12	1.05	0.34
	+ 15	- 8	1.05	0.22
	- 10	- 7	1.73	0.49
	- 1	- 3	1.14	0.37
Ascorbic acid* 375mg kg^{-1}				
- Responders [b]	+ 55	- 26	1.49	0.49
	+ 20	- 23	1.50	0.48
	+ 25	- 25	1.06	0.32
	+ 27	- 13	1.08	0.39
- Non responders	- 1	- 21	0.95	0.33
	- 4	- 29	1.17	0.30
	- 5	- 12	1.49	0.33
	- 10	- 19	1.30	0.30
Ascorbic acid 750 mg kg^{-1}	+119	- 50	1.42	0.33
	+ 19	- 29	1.38	0.21
	+114	- 48	-	-
Ascorbic acid 750 mg kg^{-1} + Vitamin E 110mg kg^{-1}	+ 37	- 26	0.07	0.00
	+ 60	- 43	0.05	0.02
	+ 75	- 44	-	-

* Microsomes prepared 5 days after last injection.
a v b, $p < 0.05$

weight loss, particularly at the high dose of ascorbic acid. However, in this group of rats, when ascorbic acid treatment was stopped, the plasma BR concentration was observed to decrease in spite of the continued weight loss. The mechanism of the toxic effect of ascorbic acid at the high dose, would appear to be different from that reported by Pohjanvirta et al, (1990) who found that toxins elevated LPO. Based on our dietary studies, an elevation of LPO would be expected to be accompanied by a decrease in plasma bilirubin.

It can be postulated that *in vivo* free radical mediated destruction of BR occurs in intra and extra cellular fluids. Vitamin C, which has an antioxidant effect in an aqueous medium, could then act by preventing free radical mediated destruction of BR so that an increase in plasma BR occurs. The rats that responded to the intermediate dose of vitamin C provide evidence in favour of this hypothesis. Although these experiments are not conclusive, they do raise the possibility that ascorbic acid has important aqueous antioxidant properties at high doses which are not toxic.

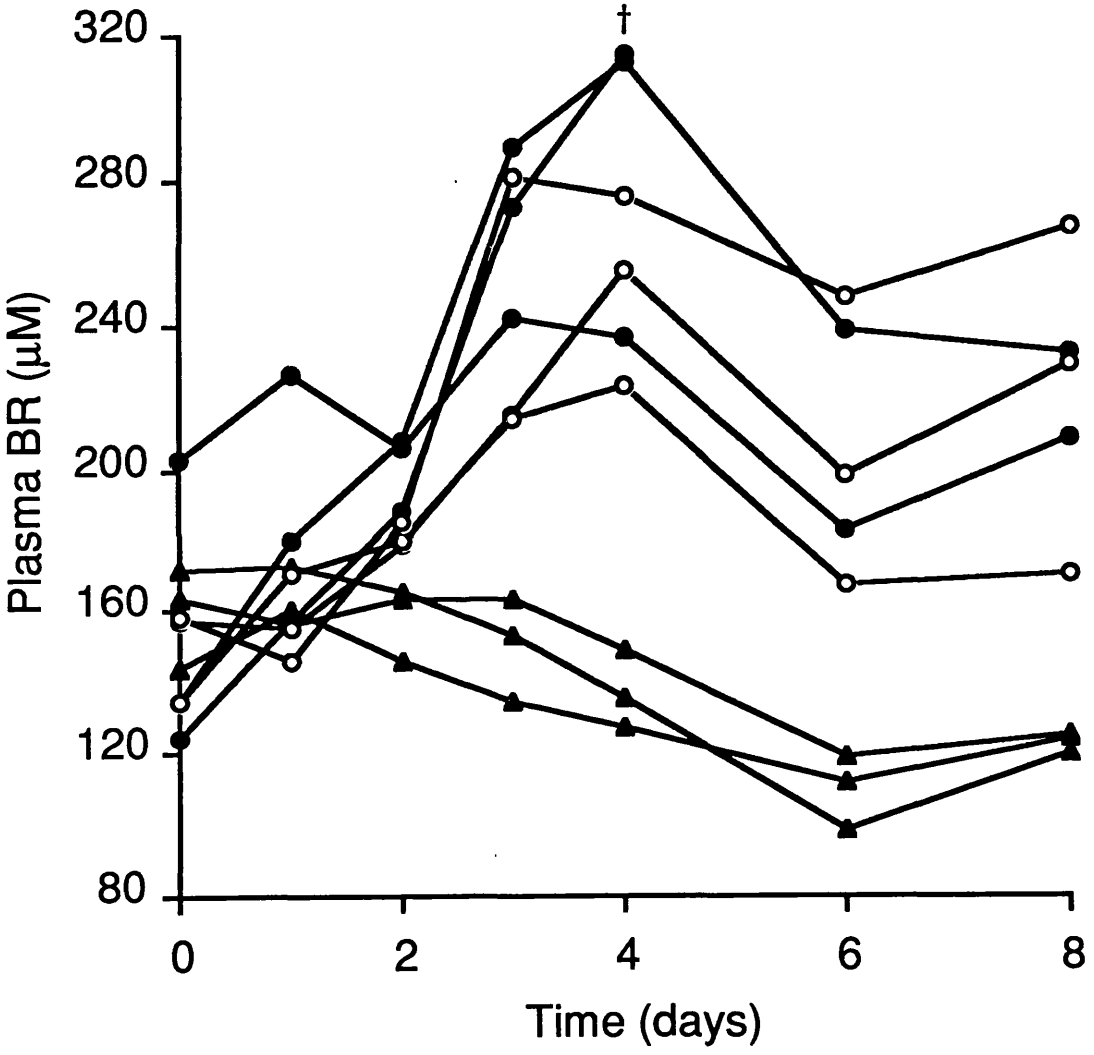
5.5 FASTING HYPERBILIRUBINAEMIA

5.5.1 INTRODUCTION

Fasting unconjugated hyperbilirubinaemia is most striking in patients with a deficiency of hepatic bilirubin uridine diphosphate glucuronyl transferase activity and in the Gunn rat.

It has been postulated that the hyperbilirubinaemia of fasting may be due to the withdrawal of dietary lipid (Gollan et al, 1979). It was therefore the purpose of this study to investigate the effect of fasting on microsomal LPO activity. It has been shown that Gunn rats fed a lipid free diet for 2 weeks, produced complete inhibition of microsomal LPO activity concurrent with a decreased peroxidizability of the microsomal membrane (DBI). Thus fasting would be expected to produce

Effect of Ascorbic Acid and Ascorbic Acid + Vitamin E on Plasma Bilirubin in Gunn Rats



Three groups of rats received intraperitonealy (i) saline (0.9% w/v NaCl) on days 0, 1 and 2 (▲-▲), (ii) ascorbic acid (750 mg kg^{-1}) on days 0, 1 and 2 (●-●); one rat died on day 5 (iii) vitamin E (110 mg kg^{-1}) on day 0 and ascorbate (750 mg kg^{-1}) on days 0, 1 and 2 (o-o). Plasma bilirubin was determined from tail vein blood on days 0,1,2,3,4,6 and 8 and plotted for each rat.

the same effects unless the hyperbilirubinaemia is produced by a different mechanism.

5.5.2 EXPERIMENTAL

Gunn rats (232-289g) were divided into 2 groups of 4 animals. Initial plasma bilirubin concentrations were determined from samples obtained by cardiac puncture under halothane anaesthesia. The experimental group was fasted for 48 h and allowed free access to water whereas the control group received the standard diet and water as usual. After 48 h, both groups of rats were sacrificed and plasma BR and hepatic microsomal LPO and BRD activities were determined.

5.5.3 RESULTS (Table 5.12)

All animals in the fasted group lost weight (mean loss ± SEM = 39 ± 1.7g), while 3 out of 4 rats in the control group gained weight after 48 h (mean 5.5 ± 3.1g); the fourth rat had a small loss in weight.

The mean liver weights expressed as percentage of the total body weights in the 2 groups were 2.5 ± 0.11% for fasted animals and 3.3 ± 0.1% for controls.

All the animals in the fasted group showed an increase in plasma BR, (mean increase ± SEM = 27 ± 10%), in 2 out of 4 animals the increase was more than 30%. The animals in the control group all showed a slight decrease in plasma BR (-12 ± 5.4%) however this was significant in only 1 animal.

The mean microsomal LPO activities for the fasted and control groups were 1.74 ± 0.1 and 1.55 ± 0.1 nmol min^{-1} mg $prot^{-1}$ respectively. Bilirubin degradation activities for the fasted and control groups were 0.41 ± 0.03 and 0.36 ± 0.01, respectively. Microsomal DBI values were determined in 2 fasted animals (mean = 130) for comparision with the standard diet microsomal DBI (mean = 125, n =

7).

5.5.4 DISCUSSION

The results confirm previous findings that fasting elevates plasma BR in animals with a deficiency of hepatic bilirubin uridine diphosphate glucuronyl transferase activity (Gollan et al, 1979, Whitmer & Gollan, 1983). However, the microsomal LPO activity in the fasted group was marginally higher than that in the control group. This is in contrast to the results obtained in the dietary experiments with rats fed a lipid free diet where an increase in plasma BR was associated with low microsomal LPO and BRD activities (Figs 5.1 & 5.2).

Thus it may be concluded that the mechanism of hyperbilirubinaemia as a result of fasting is different from that produced by lipid withdrawal. This is supported further by the fact that the DBI values for the fasted animals are comparable to the non-fasted controls, which is again in contrast to the rats maintained on a 0% lipid diet whose DBI values were significantly lower than the rats on the standard diet (Table 5.5.II).

5.6 EFFECT OF β-NAPHTHOFLAVONE ON PLASMA BILIRUBIN AND MICROSOMAL LIPID PEROXIDATION AND BILIRUBIN DEGRADATION ACTIVITIES IN GUNN RATS.

5.6.1 INTRODUCTION

It has been shown that when inducers of the microsomal mixed-function monoxygenases, TCDD (Kapitulnik & Ostrow, 1977, De Matteis et al, (1989) or β-naphthoflavone (Cardenas-Vasquez et al,1986), were injected into Gunn rats, a decrease in plasma bilirubin concentration occured. As a result of these findings, it was proposed that microsomal cytochrome P_{448} dependent monoxygenase may be involved in the catabolism of bilirubin.

TABLE 5.12 EFFECT OF FASTING ON PLASMA BILIRUBIN AND HEPATIC MICROSOMES LPO AND BRD ACTIVITIES

	Change in body wt (g)	BR conc (μM)		% change in BR conc	Microsomal activities (nmol min^{-1} mg $prot^{-1}$)	
		Initial	48h		LPO	BRD
Fasted Group						
1	- 35	118	153	+ 30	1.95	0.45
2	- 42	123	190	+ 54	1.81	0.45
3	- 37	117	130	+ 11	1.63	0.36
4	- 41	149	157	+ 13	1.55	0.36
Mean ± SEM	- 39.0 ± 1.7			+ 27.0 ± 10.0	1.74 ± 0.09	0.41 ± 0.03
Control Group						
1	+ 12	128	114	- 10	1.78	0.36
2	+ 5	105	77	- 27	1.47	0.39
3	+ 7	113	109	- 3	1.38	0.36
4	- 3	114	107	- 6	1.58	0.33
Mean ± SEM	+ 5.5 ± 3.1			- 12.0 ± 5.4	1.55 ± 0.09	0.36 ± 0.01

Others have shown that TCDD can induce hepatic microsomal lipid peroxidation (Pohjanvitra et al, 1990). It was therefore of interest to determine whether induction of microsomal mixed function monoxygenases, decreased plasma bilirubin concomitant with an increase in microsomal lipid peroxidation activity. The previously reported decrease in plasma bilirubin obtained with β-naphthoflavone (Cardenas-Vasquez et al, 1986) was therefore tested to see whether it was associated with hepatic microsomal lipid peroxidation activity in the Gunn rat. β-naphthoflavone was used as an alternative to TCDD because TCDD is a highly toxic drug and facilities for its use were not available.

5.6.2 EXPERIMENTAL

A suspension of β-naphthoflavone in corn oil (8 mg ml^{-1}) was prepared by heating in a 90°C water bath in a glass stoppered tube. Two groups of 4 male Gunn rats from the old colony (250-350g), maintained on the standard diet, received i.p. either 40 mg kg body $wt.^{-1}$ β-naphthoflavone (experimental group) or an equivalent volume of corn oil (control group). Rats were injected on three consecutive days and sacrificed on the fourth day when hepatic microsomes were prepared. Initial and final blood samples (0.5-0.75 ml) were obtained by cardiac puncture under halothane anaesthesia for plasma bilirubin determinations. Microsomal lipid peroxidation activity was determined.

5.6.3 RESULTS AND DISCUSSION

Animals in the control group had a mean gain in weight of +6g while those in the experimental group had a mean loss of -9g. Results (Table 5.13) show that both the corn oil and β-naphthoflavone injected groups produced a decrease in plasma bilirubin concentration. The mean decrease was three times greater in the β-naphthoflavone treated group.

The corn oil vehicle alone, was expected to produce a decrease in plasma bilirubin

(see dietary studies above), however a large drop was not observed in this study most probably because of the duration of the study and the mode of administration of the corn oil. The larger decrease in plasma bilirubin concentration in the experimental group is therefore due to the effect of the inducer rather than the corn oil alone.

Microsomal lipid peroxidation activity in the β-naphthoflavone treated group was lower than in the control group but the difference between the two groups does not reach significance. These findings were in contrast to previous findings by Pohjanvitra (1990) who showed that administration of a specific inducer of cytochrome P_{448} (TCDD), increased hepatic microsomal lipid peroxidation in Long-Evans rats but not in Han-Wistar rats; the reason for this strain difference has not been ascertained. These authors concluded that lipid peroxidation mainly arises as a secondary phenomenon in TCDD toxicity. A likely explanation for this discrepancy is that the dose of β-naphthoflavone selected for the present study was not sufficient to cause toxicity. Not only were the dose and duration of the studies different, but the actual inducers used were also different. It seems that although the dose and duration of the study was adequate to observe a decrease in plasma bilirubin concentration in this study, a longer exposure to the inducer may be required to observe significant changes in microsomal lipid peroxidation activity. β-naphthoflavone is not as potent an inducer as TCDD and may therefore require a longer time and a higher dose in order to observe changes in microsomal lipid peroxidation activity. The antioxidant status of the animals at the time of study could also contribute to differences in microsomal lipid peroxidation activities.

TABLE 5.13 EFFECTS OF β-NAPHTHOFLAVONE (40mg kg^{-1} Body wt) ON PLASMA BR AND MICROSOMAL LPO ACTIVITY

Group	n	Plasma BR (μM) Initial	Final	% decrease	LPO Activity (nmol min^{-1}) mg $prot^{-1}$)
Corn oil	4	138 (130-145)	131 (117-157)	5.1	1.75 ± 0.28
β-Naphtho-flavone	4	127 (106-155)	106 (84-128)	16.5	1.37 ± 0.29

Plasma BR values are means, ranges given in parenthesis. LPO activities are means ± SEM.

CHAPTER 6

DISCUSSION

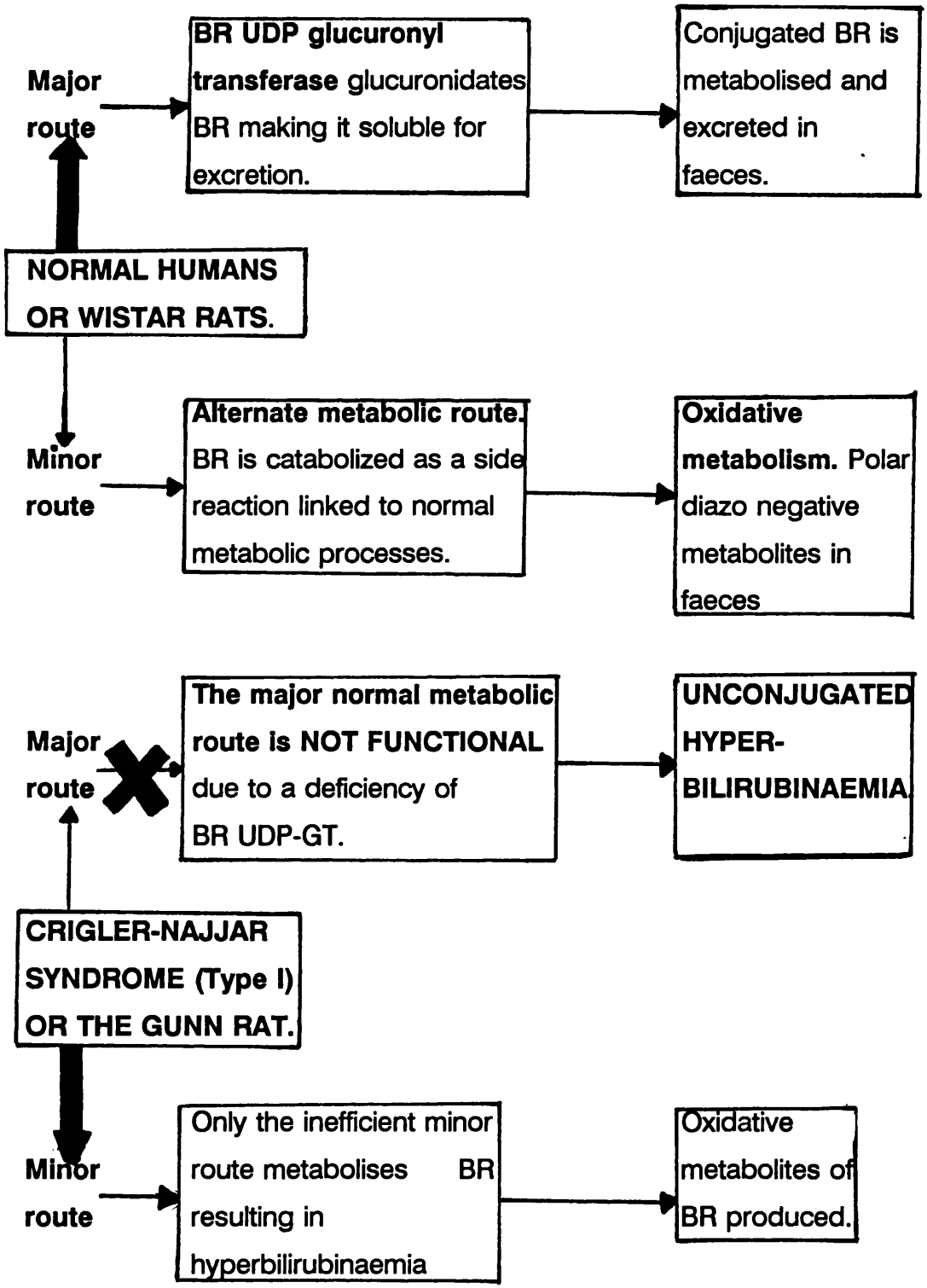
This work has investigated the problem of unconjugated hyperbilirubinaemia in the Gunn rat caused specifically by impaired conjugation of the pigment due to a congenital defect. In humans, if untreated, unconjugated hyperbilirubinaemia results in neurological dysfunction as the result of bilirubin encephalopathy. Some clinical abnormalities associated with prolonged hyperbilirubinaemia are ataxia, retarded growth and development in the neonate.

Investigations of an alternative pathway for bilirubin metabolism over the past 30 years have shown it to be an inefficient mechanism which is likely to involve an oxidative route of bilirubin degradation. The fact that the elimination process is inefficient suggests a non-specific mechanism that may be a part of normal metabolism. Support for the hypothesis that the alternative pathway is a part of normal metabolism is indicated in the present studies where similar microsomal lipid peroxidation and bilirubin degradation activities were found in Wistar and Gunn rats. It would have been expected that if a specific alternative pathway was responsible for bilirubin degradation and operated via the oxidative microsomal enzyme system, its activity would have been greater in the jaundiced Gunn rat than in the non-icteric Wistar rat.

Under normal conditions, there may be two mechanisms for the elimination of bilirubin; a major route involving bilirubin glucuronidation and a minor less efficient route. In unconjugated hyperbilirubinaemia, only the minor route would be functional (Fig 6.1).

A number of treatments for unconjugated hyperbilirubinaemia have been explored and have already been discussed (Chapter 1), however, many of them have proved to be cumbersome and impractical for life long use while others are still

FIG 6.1 HYPOTHESIS FOR AN ALTERNATIVE PATHWAY FOR BILIRUBIN METABOLISM



The alternative pathway for BR metabolism is non-specific, inefficient and yields polar diazo negative metabolites; an oxidative mechanism is suggested.

under investigation. It would therefore be important to be able to specifically and safely stimulate the alternative pathway.

Lipid peroxidation, an oxidative mechanism that occurs as a part of normal metabolism, has been shown in these studies using Gunn rat hepatic microsomes, to oxidize bilirubin to diazo negative products. It therefore seems a likely minor route for bilirubin metabolism *in vivo* although the rates of LPO *in vitro* are much greater than would be expected *in vivo*. With this proviso in mind, further studies have therefore investigated the effect of this oxidative mechanism on bilirubin degradation *in vitro* and *in vivo*. Plasma bilirubin concentration was used as an index of bilirubin metabolism *in vivo* while microsomal lipid peroxidation was used as an indicator of membrane peroxidizability. The *in vitro* studies showed a strong correlation between hepatic microsomal lipid peroxidation and bilirubin degradation activities.

Enzymatic lipid peroxidation was inhibited by heating microsomes while the addition of glutathione or high concentrations of ascorbic acid inhibited both enzymatic and non-enzymatic LPO; concurrent inhibition of bilirubin degradation was obtained. The mechanism of lipid peroxidation is still uncertain but in the system studied it has been shown that neither hydroxyl radicals, superoxide radicals or hydrogen peroxide are involved; this confirms previous findings (Pederson & Aust 1975) where perferryl ions were proposed as the most likely initiators of lipid peroxidation.

The *in vivo* studies showed that dietary modulation of plasma bilirubin could be related to changes in microsomal lipid peroxidation activities. The differences in microsomal lipid peroxidation activity did not however correlate with microsomal cytochrome P_{448} activity, which was found to be similar in all three dietary lipid groups (0%, 8% and 25% corn oil). Furthermore, no significant degradation of bilirubin occurred when microsomes were aerobically incubated with NADPH without Fe/ADP these conditions are suitable for cytochrome P_{450} activity, but do

not efficiently initiate lipid peroxidation. Also, even when microsomal enzymes were destroyed by heat-treatment, the diet-induced differences in non-enzymatic microsomal lipid peroxidation activity persisted.

The effect of dietary fats on benzo (a) pyrene metabolism, which is used as an index of cytochrome P_{448} activity, was previously investigated using rat liver microsomes and found to be related to the diet and fatty acid composition of the membranes (Wills, 1983) . These studies also demonstrated that microsomes from rats fed diets rich in polyunsaturated fatty acids were more effective in metabolising benzo (a) pyrene than microsomes from rats fed diets containing either less polyunsaturated fatty acids or those containing mainly saturated fats. The concentration of cytochrome P_{450} in the liver was also affected by the type and quantity of lipid; it was increased after feeding diets rich in polyunsaturated fats compared with fat free diets. This change in cytochrome P_{450} concentration has been postulated by Wills as a possible mechanism for the increased oxidation of compounds such as benzo (a) pyrene and aminopyrene. It is possible that changes in fatty acid composition of membrane phospholipids may alter the configuration of active enzymes in the membrane. Another alternative is that the free radicals formed during the peroxidation of polyunsaturated phospholipid fatty acids, or from the fatty acids released from phospholipids, could be responsible for the increased oxidation. In contrast to Wills' finding, the high fat diet in our study did not increase cytochrome P_{448} activity and needs further investigation.

The dietary changes in lipid peroxidation activity in our study could, however, be correlated with the polyunsaturated fatty acid content of the liver microsomal membrane (Chapter 5). The evidence regarding differences in vitamin E concentration in microsomes from rats fed the different diets was less conclusive. There was a tendency towards a higher vitamin E concentration in microsomes with low lipid peroxidation activity, while in microsomes with a low vitamin E concentration lipid peroxidation activity was high. This trend seems to favour vitamin E playing an antioxidant role in the system. It can therefore be suggested

that the changes due to dietary lipid are directly related to the peroxidizability of (microsomal) membranes which in turn depends on the percentage of unsaturated lipid and the vitamin E concentration in the membrane.

Polyunsaturated fatty acids could play a very important role in the regulation of membrane structure and activity of the endoplasmic reticulum. The mechanism by which dietary polyunsaturated fatty acids might regulate the rate of oxidative metabolism in the liver is not clear. The present studies with Gunn rat liver microsomes, show that a synthetic diet rich in unsaturated fatty acids, increases the degree of unsaturation of the membrane lipids and causes greater production of free radicals *in vitro* as the result of increased lipid peroxidation. The concentration of vitamin E in hepatic microsomes may also be altered by diet, the changes are, however, more likely to result from differences in its utilization.

The use of antioxidants, both lipid soluble and water soluble was expected to confirm the role of lipid peroxidation in bilirubin metabolism in the Gunn rat, however, these data were complicated by the use of a newly established Gunn rat colony which behaved differently from the old colony. The varied response in the new colony suggests that it takes a few generations before a truly homogenous group is established. Although the rats had similar plasma bilirubin concentrations, other characteristics which were not phenotypically obvious or necessarily linked to the degree of jaundice might be expected to persist for several generations. In fact, unless rats are selectively bred for other characteristics than jaundice, it may well take several years to obtain a truly homogenous colony like the old colony which had been maintained for over 30 years.

The results from antioxidant administration showed that the lipid soluble antioxidant vitamin E did not influence plasma bilirubin, although microsomal lipid peroxidation was completely inhibited. However, the aqueous antioxidant vitamin C, could be inhibiting bilirubin metabolism although it failed to inhibit microsomal lipid peroxidation. It may, therefore, be concluded that although bilirubin metabolism is

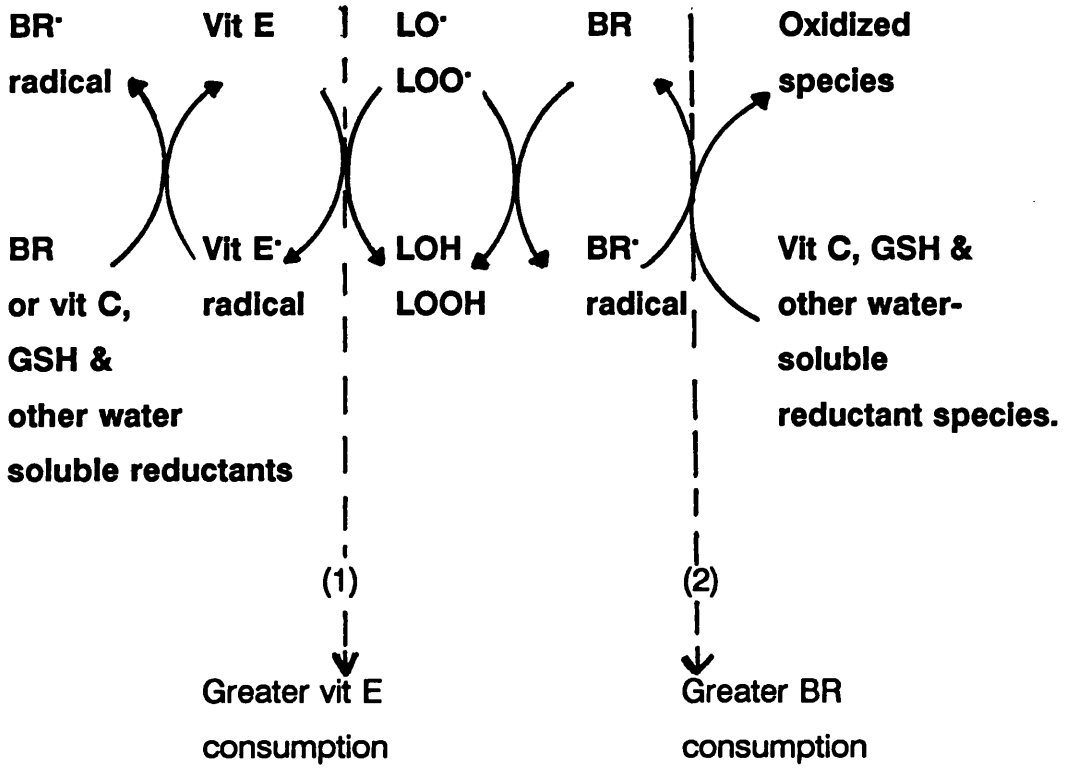
influenced *in vivo* by altering lipid peroxidation with dietary lipid, the inhibitory effect of vitamin E on lipid peroxidation does not appear to influence bilirubin metabolism. These findings support the hypothesis that bilirubin metabolism by the minor route could be occurring mainly in the aqueous phase and that the free radicals that arise as a result of lipid peroxidation (eg $L^{\cdot}$, $LOO^{\cdot}$) form only a part of this pathway. Alternatively, antioxidants in the aqueous phase may modulate rates of lipid peroxidation and thereby alter bilirubin degradation. The fact that dramatic effects on plasma bilirubin were observed in the dietary lipid study could be because free radicals produced during lipid peroxidation are directly increased or decreased by a high or low lipid diet respectively.

It is worth noting that vitamin E and bilirubin would be competing both for lipid free radicals, as they are thought to interact at the same point in the peroxidation mechanism, and for recycling systems employing water soluble reductants . A competitive effect, whereby bilirubin is selectively regenerated from its radical, could account for the lack of a vitamin E effect on plasma bilirubin *in vivo*; a speculative model is shown in Fig 6.2.

At higher concentration of vitamin E, compared to bilirubin, radical species produced during lipid peroxidation might be more favourably scavenged by vitamin E and the left hand side of the scheme in Fig. 6.2 (1) would be favoured. Vitamin E would therefore decrease bilirubin oxidation by the lipid radical species. Vitamin E can be regenerated from its radical by physiological concentrations of vitamin C, GSH and perhaps bilirubin. Bilirubin is less likely to be effective in regenerating vitamin E than the aqueous antioxidants because it is protein bound and also because it has been shown to be a poor antioxidant for membrane lipid peroxidation in this study. There may therefore be physical constraints on the molecule which may prevent it from being as effective as vitamin C and GSH.

If the concentration of bilirubin becomes relatively higher than vitamin E, the right hand side of the scheme in Fig.6.2 (2) might be favoured so that there would be

FIG 6.2 HYPOTHETICAL MECHANISM FOR THE CONTROL OF LIPID PEROXIDATION AND BILIRUBIN DEGRADATION.



Lipid radicals formed during LPO can convert BR and / or vit E to their respective radical species. These radical species can be converted back to the original molecules by either soluble reductants e.g. vit C and GSH or, in the case of vit E, possibly by bilirubin. The concentration of vit E in relation to the concentration of BR will determine which of these will be consumed to a greater extent, since both compete for lipoxyl and peroxyl radicals at the same point in the lipid peroxidation chain reaction. Other factors that may influence the LPO and BRD activities are the polar molecules vit C, GSH, NADPH and NADH, all of which may recycle vit E from its radical by a non-specific mechanism and may have the same effect on BR.

a greater consumption of bilirubin. By a similar mechanism to that for vitamin E, it is possible for bilirubin to be regenerated from its radicals, although this has not yet been demonstrated.

In summary, the scheme outlined in Fig 6.2, shows that high concentrations of vitamin E can result in decreased bilirubin consumption by competing with it in scavenging the lipid radical species. Surprisingly, a rise in plasma bilirubin was not observed when vitamin E was injected into Gunn rats, suggesting that the bilirubin is being degraded by other mechanisms. These may involve free radicals in the aqueous phase or water soluble reductants, which regenerate bilirubin from its radical. Finally, bilirubin may weakly regenerate vitamin E. Alternatively, bilirubin metabolism could depend on some other mechanism not investigated in these studies.

Albumin bound bilirubin is distributed throughout the aqueous extracellular space while bilirubin is bound to ligandin in the intracellular space. Bilirubin might therefore be susceptible to attack by free radicals in the aqueous environment, most likely extracellularly. Inhibition of this process by the aqueous antioxidant ascorbic acid at intermediate and high doses could account for the increased plasma bilirubin observed. Whereas it was most likely to have a toxic effect at the high dose, the antioxidant property of ascorbic acid was possibly demonstrated at the intermediate dose.

A combination of both types of antioxidants vitamins E and C would be expected to elevate plasma bilirubin to a greater extent than when separately administered due to inhibition of free radical attack in both the aqueous and lipid environments. This hypothesis was examined only at the high dose of ascorbic acid where this additive effect was not observed (Fig 5.7); the plasma bilirubin concentration increased, but to a lesser extent than with vitamin C alone. Further evidence using different doses of ascorbic acid, a homogenous colony of Gunn rats and other aqueous antioxidants, is required to support this hypothesis.

The interaction of the protective mechanisms *in vivo*, together with the homeostatic balance between free radical production and protective mechanisms which may vary according to the nutritional status and/or disease state, presents complications in isolating a single factor responsible for the alternate pathway for bilirubin metabolism. However, these studies have implicated a free radical mediated destruction of bilirubin *in vivo* which is non-specific and suggests, therefore, that bilirubin degradation occurs as a by- product of normal metabolism.

Free radicals arise in normal metabolism as exemplified by phagocytosis, eicosonoid synthesis, membrane catabolism and cell damage. These short lived species, if not scavenged by the normal protective mechanisms (vitamins E and C, GSH, SOD, catalase etc), could be harmful. Bilirubin posseses the requirements of a chain breaking antioxidant and has been shown under certain conditions to have such a property *in vitro* (Stocker, et al 1987). If it acts as an antioxidant *in vivo*, bilirubin itself would become oxidised, possibly to polar diazo negative products. These products have been well documented as the breakdown products of bilirubin metabolism in bilirubin glucuronyl transferase deficient animals (Schmid & Hammaker, 1963).

Whether bilirubin, a breakdown product of haem, is produced *in vivo* for an antioxidant protective role is debatable. Others (Stocker, et al 1987) have suggested that mammals have evolved an energy expensive mechanism for bilirubin production for this specific purpose. However, it is also possible that, like the alternate pathway for bilirubin metabolism, the production of bilirubin is a part of normal metabolism, and it may happen to have an antioxidant property.

It was interesting to find that in fasting hyperbilirubinaemia in the Gunn rat, the hepatic microsomal lipid peroxidation activity and the total DBI in the fasted animals was similar to that in the control animals although Pohjanvitra et al, (1990) found that fasting increased lipid peroxidation in one of two rat strains. It has been concluded (Chapter 5) that the increase in concentration in bilirubin due to fasting

occurs by a different mechanism to lipid withdrawal. It is worth noting that, to see an effect on the microsomal phospholipid fatty acid profile, prolonged fasting as used by Pohjanvitra may be required. In our study, the rats were only subjected to a 48 h fast. Further work is required to acertain the exact mechanisms involved.

Microsomal enzyme inducers such as TCDD (Kapitulnik & Ostrow, 1977, De Matteis et al, 1989) and β-naphthoflavone (Cardenas-Vasquez et al, 1986,) have been shown to decrease plasma bilirubin concentration in the jaundiced Gunn rat. This finding has been correlated with the induction of enzymes of the microsomal cytochrome P_{450} system. Since TCDD can also induce lipid peroxidation in hepatic microsomes in some strains of rats (Pohjanvirta et al, 1990), an alternate hypothesis is that the reduction in plasma bilirubin in induced animals is due to increased hepatic microsomal lipid peroxidation, although this would not be apparent in the *in vitro* assay of De Matteis et al (1989) due to the presence of EDTA. The lack of induction of microsomal lipid peroxidation by β-naphthoflavone in our studies requires further investigation; it may essentially be due to experimental design (Chapter 5).

Since dietary manipulation of lipid peroxidation *in vivo* has a marked effect on plasma bilirubin (Chapter 5) and an induction of cytochrome P_{448} has been shown to increase lipid peroxidation activity *in vitro*, an association between these two mechanisms cannot be disregarded. It is however still unclear whether these oxidative mechanisms are the most important in regulating bilirubin degradation in the Gunn rat or patients with severe unconjugated hyperbilirubinaemia.

FUTURE STUDIES

The general hypothesis that, free radical mediated bilirubin oxidation *in vivo* is responsible in part for the normal metabolism of bilirubin and completely for bilirubin metabolism in glucuronidation deficiency, requires further support. In this study, hepatic microsomal lipid peroxidation was investigated as the main free

radical mediated process that may be responsible for the alternative pathway of bilirubin metabolism. Peroxidation of the hepatic microsomal membrane lipids provided a good *in vitro* estimate of lipid peroxidation in vivo. However, a more direct index of overall free radical status is required *in vivo* to provide an accurate means of correlating lipid peroxidation and plasma bilirubin concentration. Such an index would have to account for compensatory antioxidant mechanisms that might be operating and would therefore be clinically more useful; this index requires to be developed.

From these studies, it is apparent that unless the lipid composition or antioxidant concentration in the microsomal membrane is altered, microsomal lipid peroxidation serves as a poor indicator of the free radical status *in vivo*. Neither activity of free radicals in the aqueous phase nor activity of mechanisms dependent on water soluble reductants, which might recycle radical species to the parent molecule *in vivo*, is reflected in microsomal lipid peroxidation determinations. Future studies need to consider the role of aqueous phase free radicals and reductants in the metabolism of bilirubin.

The role of free radicals in disease and toxicity will only be truly informative when their steady state concentrations can be accurately and directly measured *in vivo*. This is extremely difficult since they are short-lived species that arise in beneficial as well as harmful processes in the body. At present, electron spin resonance is the main method used to assay free radicals.

Identification of bilirubin metabolites in the *in vitro* assay, which unfortunately has been unsuccessful to date, would lead to a better understanding of the mechanism that may be involved *in vivo*. If the bilirubin metabolites formed *in vitro* were the same as bilirubin metabolites in the Gunn rat, further evidence would be provided in support of the involvement of lipid peroxidation as an alternative pathway for bilirubin metabolism. The use of either ^{14}C BR or the electron spin resonance technique with the use of suitable spin traps, may provide useful

approaches for these investigations.

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